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Effects of nanosilver and silver nitrate exposure on fathead minnows (Pimephales promelas) and zebrafish (Danio rerio)

Adam D. Hawkins
University of Mississippi

EFFECTS OF NANOSILVER AND SILVER NITRATE EXPOSURE ON FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) AND ZEBRAFISH (*DANIO RERIO*)

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By

ADAM D. HAWKINS

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ABSTRACT

Silver nanoparticles (AgNPs) due to their unique antimicrobial properties are among the most widely utilized nanoparticle. Because of the nature of the products that utilize AgNPs, environmental release of AgNPs is inevitable. At greatest risk is the aquatic ecosystem due to known toxicity of the Ag^+ ion to aquatic organisms. Our goal was to use two fish models, fathead minnows (*Pimephales promelas*; FHMs) and zebrafish (*Danio rerio*; ZFs), to study the effects of AgNPs on gill histopathology, Na^+/K^+-ATPase immunoreactivity, mucus production, bioaccumulation, particle uptake characterization, gill gene expression, and lethality in varying water parameters in comparison to the known toxicities of Ag^+ in the form of $AgNO_3$. FHMs were exposed to two nominal concentrations of $AgNO_3$ (2 and 6 µg/L), citrate-AgNPs and polyvinylpyrrolidone–AgNPs (20 and 200 µg/L; 20 nm). All silver groups had significantly higher histopathological abnormalities with citrate-AgNPs having the highest toxicity (index of 10±0.32 versus 2.4±0.6 in controls). Gill Na^+/K^+-ATPase immunoreactivity was decreased by silver. Control fish produced mucus consistently while silver-treated fish initially produced significantly more mucus but this production was reduced below control concentrations by 96 hr of silver exposure. Silver accumulation was quantitated by inductively coupled plasma mass spectrometry (ICP-MS) after 96 hr $AgNO_3$ and AgNPs exposure. Despite higher exposure concentrations, AgNPs accumulated at lower concentrations than $AgNO_3$ in the gill, skin, and liver with none detected in the

brain. AgNPs accumulated in the GI tract more than $AgNO_3$ indicating a possible unique biological target of AgNP exposure. To understand this further, Field-Flow-Fractionation coupled to ICP-MS (FFF-ICP-MS) was utilized to characterize particles in GI tract and gill tissue. Particles were sized between 26-70 nm in GI and 27-30 nm in gill. FHM gill microarrays were performed to compare alterations in gene expression between AgNPs and $AgNO_3$. Analysis revealed 110 commonly differentially expressed genes between all silver treatments compared to control with 185, 423, and 615 unique differentially expressed genes in $AgNO_3$, PVP-AgNPs and citrate-AgNPs, respectively. ZF larvae were exposed 48 hrs to AgNPs and $AgNO_3$ in increasing environmentally relevant concentrations of dissolved organic carbon (DOC) and higher DOCs decreased silver lethality. Overall, while AgNPs displayed some unique particle effects, the majority of the toxicities were consistent with Ag^+ release.

DEDICATION

This work is dedicated to my family, my girlfriend Katie, my friends and fellow labmates in Environmental Toxicology Research Program.

LIST OF ABBREVIATIONS

Bovine serum albumin (BSA)

Carbon nanotubes (CNTs)

Citrate-coated silver nanoparticles (citrate-AgNPs)

Clean Water Act (CWA)

Consumer Products Inventory (CPI)

Differentially expressed genes (DEGs)

Dissolved organic carbon (DOC)

Dynamic light scattering intensity (DLS)

Effective diameters (ED)

Engineer Research and Development Center- Environmental Laboratory (ERDC-EL)

European Commission (EC)

Fathead minnows (FHMs)

Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)

Field-Flow-Fractionation coupled to Inductively Coupled Plasma Mass Spectrometry (FFF-ICP-MS).

Hours post fertilization (hpf)

Hematoxylin and eosin staining (H&E)

Hydrodynamic diameter (HD)

Inductively coupled plasma mass spectrometry (ICP-MS)

Ingenuity Pathway Analysis (IPA)

Institutional Animal Care and Use Committee (IACUC)

International Humic Substances Society (IHCC)

National Nanotechnology Initiative (NNI)

Organic light-emitting diodes (OLEDs)

Polyvinylpyrrolidone – coated silver nanoparticles (PVP-AgNPs)

Major histocompatibility complex (MHC)

Median lethal concentration (LC_{50})

Moderately hard water (MHW)

National Pollutant Discharge Elimination System (NPDES)

Self-assembled monolayers on mesoporous supports (SAMMS)

Silver nanoparticles (AgNPs)

Standard error of the mean (SEM)

Transmission electron microscopy (TEM)

United States Department of Agriculture-National Sedimentation Laboratory (USDA-NSL)

United States Environmental Protection Agency (USEPA)

Water-quality standards (WQS)

Water-quality criteria (WQC)

Zebrafish (ZF)

Zero-valent iron nanoparticles (nZVI)

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CHAPTER 1. INTRODUCTION

1. Nanoparticles and silver nanoparticles (AgNPs)

1.1 Nanoparticle definition

Nanoparticles are defined by the National Nanotechnology Initiative (NNI), a program established by the U.S. Government to coordinate research and development efforts in nanoscale science, engineering, and technology among 26 federal agencies, as:

1. Man-made particles at the atomic, molecular, or macromolecular levels, in the length scale of approximately 1-100 nanometer (nm) range in any direction.

2. The particle has structures, devices, and systems that have novel properties and functions as a result of their small and/or intermediate size. (USEPA 2007)

The European Commission (EC) expands that definition to include naturally occurring and incidentally created particles as well as mixtures that contain at least 50% of particles with a size less than 100 nm in any external dimension. The EC definition also allows for a broadened description in specific cases warranted by increased concern for the environment, health, safety or competitiveness where the 100 nm in any external dimension threshold of 50% may be replaced by a threshold between 1 and 50 % (Commission 2011).

1.2 Origins and increased use of nanotechnology

The vision of nanotechnology is credited to physicist and Nobel Laureate Richard Feynman. In a lecture at the Californian Institute of Technology in 1959, he

presupposed that new technology will arise from the ability to manipulate atoms and molecules (Feynman 1960). Feynman's vision was not realized until 1990 when the technology, in the form of a Scanning Tunneling Microscope (Binnig et al. 1982), was first utilized to move individual atoms (Maynard 2007). Eigler and Schweizer used 35 single xenon atoms on a nickel surface to create an IBM logo (Eigler and Schweizer 1990). As technology progressed, matter was manipulated at the near-atomic scale to alter properties of a material and create useful structures (Maynard 2007).

Carbon nanotubes (CNTs) are one such creation, discovered in the early 1990s (Bethune et al. 1993; Iijima 1991), in the beginning of the nanotechnology timeline. The synthesized CNTs demonstrated improved qualities over larger carbon based materials, such as graphite, including: greater tensile strength; ability to conduct electricity; and other desirable thermal and mechanical properties (Ball 1999; Ball 2001; Arepalli et al. 2001). Current work in nanotechnology yields new materials that improve technologies or changes the characteristics of bulk material to more useful forms (Dowling et al. 2004).

The promising discoveries and investments in nanotechnology led to a boom in the availability of consumer products that contain nanoparticles. Indicators of technology development include patent applications, which rose from 1,197 to 12,776 from the 2000 to 2008. The final value of nanotechnology products available on the market rose from ~ $30 billion to ~ $200 billion in the same time period (Roco 2011). Along with the rapid growth and potential benefits of nanotechnology comes a need to understand and minimize the potential risks involved with rapid integration of nanoparticles into products (Maynard et al. 2006).

The Consumer Product Inventory (CPI) (Nanotechnologies 2014) established by the Project on Emerging Nanotechnologies (http://www.nanotechproject.org) is the most comprehensive list of known consumer products using nanotechnologies. Known products with nanoparticles have increased exponentially since the CPI began in 2005. The first year only 58 products were known to have nanoparticles, whereas in 2013, that number had increased to 1628. The greatest number of consumer products in which particles are used are primarily health and fitness products. Of the 788 products in this category, 292 are personal care products, 187 are clothing, and 154 are cosmetics. Nanosilver is among the most used nanoparticle with 23% of all known product with nanotechnologies containing AgNPs (*Consumer Products Inventory* 2014).

1.3 Benefits of nanotechnology

Nanotechnology is not only applied to the creation of new novel products, it is also been utilized in the improvement of existing products and technologies. Nanotechnology has also advanced technologies in building materials, increased sustainability of consumer products, and is utilized in medical technology.

1.3.1 Nanoparticles in everyday products

Nanoparticles are being increasingly used in common products to make them more durable, lighter and so that fewer resources are needed in manufacturing. Nanoparticles are found in many items commonly used in households such as: batteries, air and water purifiers, cleaners, paints, and sealing products. One product with common nanoparticle application is textiles. Nanotechnology is used to increase water repellence, UV-protection, anti-bacterial properties, anti-static properties, and wrinkle resistance (Wong et al. 2006). As the technology becomes cheaper and

industrialization continues in areas of textile manufacturing, nanoparticles are predicted to be increasingly applied to textiles (Ngô and Van de Voorde 2014).

Nanotechnology is also applied in the food industry. Nanoparticles are being used in food storage containers and packaging to prevent the growth of bacteria and to keep food fresh and safer longer. Biosensors at the nano-level are also utilized to detect contaminates such as bacteria or pesticides to prevent distribution for consumption (Prakash et al. 2013). Nutraceuticals are being engineered on the nano-scale to improve delivery of nutrients in the body (Sozer and Kokini 2009).

The cosmetic industry was among the earliest industries to incorporate nanotechnology into products. Many products amalgamate TiO_2 nanoparticles into products as protection from ultraviolent rays (Wiechers and Musee 2010). Due to the unique properties nanoparticles typically exhibit over their larger bulk counterpart, particles are also used to provide greater clarity, color, coverage, and stability. Nanoparticles also contribute cleansing and anti-microbal properties to shampoos, lotions, and skin cleansers (Raj et al. 2012).

1.3.2 Nanoparticles in electronic devices

Nanotechnology is already incorporated into the electronics that that 91% of Americans use daily in cell phones or other electronic devices (http://www.pewinternet.org/2013/06/05/smartphone-ownership-2013/). Nanoscale developments have yielded faster, smaller and more energy-efficient computer processors and transistors. Many computers, phones, and TVs are also intergrading nanotechnology into screen displays in the form of nanostructured organic light-emitting diodes (OLEDs) which are offer better color saturation, viewing angles, and decreased

energy requirements.

Portable electronic devices also benefit from nanotechnology incorporated into batteries. These batteries offer longer life cycles, reduced charging times, smaller and lighter form-factor, and reduced fire risks. Also included are nanoscale improved flash memory that allow greater storage capacity, reduce energy requirements and faster loading times (Chung et al. 2010).

1.3.3 Environmental benefits

1.3.3.1 Environmental remediation

New nanotechnologies are also being developed for the eradication of pollutants from the environment and water sources. One such application is the creation of self-assembled monolayers on mesoporous supports (SAMMS). SAMMS are created for the adsorption of contaminants from the desired media by the addition of substances with adsorptive properties to a ceramic support frame. The created SAMMs offer a large surface area and are made to accommodate the removal needs of the contaminated area (Zhang 2003). SAMMs technology is beneficial in the removal of actinides (Lin et al. 2005), and ions from heavy metals including cadium, cobalt, copper, chromium, lead, nickel, zinc, and manganese (Yantasee et al. 2003).

Nanotechnologies are also being developed for use to purify drinking water for the 1.1 billion people in the world without access to a potable water supply (Hillie and Hlophe 2007). Filtration with nanoparticles has proven to be more effective than conventional filtration because carbon nanotube filters more effectively removed bacteria and viruses (Grimshaw 2011). Zero-Valent Iron Nanoparticles (nZVI), which have higher reactivity then their larger counter parts, have been useful in breaking down

many common contaminants such as inorganic and heavy metal ions, polychlorinated biphenyls, and perchlorate (Theron et al. 2008).

1.3.3.2 “Green” technologies

In addition to the previously stated efforts that increase energy efficiency and reduce waste, strides are being made in the creation of renewable energy with nanotechnology. Photovoltaic solar panels created as thin nano-engineered sheets offer flexibility, durability, and cheaper manufacturing costs over conventional panels. This technology also allows for the application of panels to smaller devices and portable devices (Serrano et al. 2009).

1.4 Nanosilver

Silver has been used for treatment of burns and bacterial infections for hundreds of years (Klasen 2000). Silver’s use as an antimicrobial agent declined as antibiotics were introduced, however as bacteria increasingly develop antibiotic resistant strains interest in silver’s antimicrobial potential has renewed. Nanoparticle silver, with increased surface area and reactively relative to bulk silver, is appealing for various medical applications including wound dressing and coatings on medical devices (Rai et al. 2009). Applications to other surfaces, such as textiles, are increasing as AgNPs have shown an antibacterial ability against *Escherichia coli* and *Staphylococcus aureus* on such surfaces (Perelshtein et al. 2008).

Due to the antimicrobial properties of AgNPs, they are among the most widely used nanoparticles with applications in clothing to prevent odor, food storage containers to reduce bacterial build up, children’s toys to fight the spread of disease, and washing machines for the cleaner clothes by prevention of bacterial build up (*Consumer*

Products Inventory 2014). Normal use of many products containing AgNPs includes washing which can release silver into water in high concentrations. For example, an athletic shirt washed in 500 mL of tap water released 27 µg of silver (Benn et al. 2010). Due to a lack of methods for quantification of AgNPs at environmentally relevant concentrations, a knowledge gap exists in the actual measured environmental concentrations of AgNPs (Von der Kammer et al. 2012). However some tools such as probabilistic modeling which incorporates AgNP production, release, and flow have been used to calculate theoretical environmental concentrations. One model predicated silver concentrations of 0.02 µg/L in sewage treatment effluent and 0.0094 µg/L concentrations in surface water (Sun et al. 2014). As AgNP are included in more products environmental concentrations could increase into a more troublesome range.

2. Known toxicity of silver

Silver in the form of the Ag^+ ion is known to be the second most toxic metal after mercury to fish. The toxicity from silver exposure is derived from the binding of the Ag^+ ion to the gill resulting in disruption in Na^+ and Cl^- uptake by inhibition of Na^+/K^+-ATPase. This results in reduction of Na^+ and Cl^- plasma levels, inability to control fluid levels, increased ammonia concentrations, alterations of fish gill structure and ultimately cardiovascular failure (Morgan et al. 1997; Wood et al. 1999; Bury et al. 1999).

Due to the acute lethality of the Ag^+ ion, acute toxicity of has been well characterized in many species (Table 1.1) with LC_{50}s ranging from 9-30 µg/L in a variety of large fish species. Considerably less is known about the chronic effects of Ag^+ ion exposure to fish. Longer term exposures have shown similar mechanisms with

disruption of ionoregulation in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to as little 1.0 µg/L from fertilization to swim-up (Brauner and Wood 2002; Brauner and Wood 2002). Other endpoints of chronic silver exposure were mortality, increased time to hatch, reduced growth, and increased larval ammonia levels (Brauner and Wood 2002; Davies et al. 1978; Nebeker et al. 1983). The resulting reduction in growth could be due to diversion of metabolic resources to restore ionoregulation or metal detoxification including increased production of metallothionein (Andren and Bober 2002).

Table 1.1. Acute lethality of Ag^+ ion in multiple fish species.

Fish Species	LC_{50} (µg/L)	Reference
mosquitofish (*Gambusia affinis*)	23.5	(Diamond et al, 1990)
flagfish (*Jordanella floridae*)	9.2	(Lima et al,1982)
bluegill (*Lepomis macrochirus*)	31.7	(Diamond et al, 1990)
coho salmon (*Oncorhynchus kisutch*)	12.5	(Buhl and Hamilton, 1991)
rainbow trout (*Oncorhynchus mykiss*)	10.9	(Nebeker et al, 1983)
steelhead trout (*Oncorhynchus irideus*)	9.2	(Nebeker et al, 1983)
Chinook salmon (*Oncorhynchus tshawytscha*)	33	(Smith and Carson, 1977)
fathead minnow (*Pimephales promelas*)	5.3-20	(Leblanc et al, 1984)

While the acute lethality of released ions from AgNPs can be of great consequence to aquatic organism, the lack of comprehensive knowledge about chronic toxicity is magnified in regards to AgNP exposure. AgNPs have shown lesser toxicity when acute lethality is concerned based on total silver dose, but ramifications about chronic exposure due to slow release of Ag^+ ions are not well characterized. Also, AgNPs could accumulate in different tissues, reach different biological targets, or interact directly with tissue leading to differing toxicities of AgNP exposure.

3. Regulatory framework of silver in the environment

3.1 Regulation of ionic silver

Silver is regulated under the Clean Water Act (CWA) by the USEPA and individual states in the United States. The CWA requires the USEPA to establish and achieve safe levels of pollutants in surface waters. In compliance with the CWA, the USEPA must first establish water-quality standards (WQS) to protect aquatic organisms by incorporating acute lethality data and chronic data to prevent sublethal effects. The acute lethality criterion is defined by establishment of one-half of the 95^{th} percentile for LC_{50}s of different aquatic organisms and is expressed as concentration that should not be exceeded for more than one hour every three years (Stephen et al. 1985). Because a lack of data exists about chronic Ag^+ toxicity, no national chronic WQS has been established and regulation relies on the currently established acute WQS criteria for silver of 3.4 µg/L (Andren and Bober 2002).

States then use established WQS to create water-quality criteria (WQC) that are

enforceable standards that establish the maximum amount of a pollutant in water. States must also identify waters that currently do not meet WQC and develop total maximum daily loads (TMDLs) for a pollutant. In order to prevent pollution levels above WQC and TMDLs, pollution discharge is not allowed in water from a point source unless release is able to be achieved without violation of established criteria and a national pollution discharge elimination system (NPDES) permit is obtained (Andren and Bober 2002).

3.1 Regulation of nanosilver

While nanosilver release from point sources must comply with current regulations for silver release, AgNPs are also currently regulated under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Updates to section 6(a)(2) of FIFRA were established to better assess and regulate inclusion of nanotechnology in pesticides in 2011. Changes include declaration of presence of nanoparticles in currently or newly registered pesticides (Kalil 2011). These changes are especially applicable to AgNPs. Because of AgNPs' antimicrobial properties, products that traditionally would not be considered a pesticide, including normally used consumer goods, could now be regulated under FIFRA at the discretion of the USEPA and thus requiring registration of the products and assessment of environmental and human health risk.

4. Model organisms

4.1 Fathead minnows (*Pimephales promelas*)

FHMs were utilized in the present study because they are found in a wide range

of aquatic ecosystems where AgNPs could be released and are able to withstand changes in environment. The USEPA also utilizes FHMs as one of the main fish used acute toxicity testing. FHM testing has resulted in a large USEPA database with known acute toxicity established in 617 industrial chemicals (http://www.epa.gov/ncct/dsstox/sdf_epafhm.html).

4.2 Zebrafish (*Danio rerio*)

Zebrafish were utilized as a model fish because their developmental stages are well defined. Zebrafish also produce a large quantity of offspring with a short generation time. The small size of larval zebrafish allow them to be inserted into 96 well plates for early life stage studies which reduces waste and resources needed for testing. The large number of offspring produced and small larval size also are conducive to higher throughput testing.

5. Hypotheses and aims

Because of the known toxicity of the Ag^{+}, we hypothesized that the toxicity of AgNPs is mostly derived from the ionic portion but because of different accumulation, ion release over time, and direct particle interaction with biological surfaces AgNPs will create unique particle toxicities.

Aim 1. Identify changes in gill histopathology after exposure to silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$).

Aim 1.1. Record occurrences of specific gill histopathological alterations after exposure to AgNPs of $AgNO_3$ and utilize a scoring matrix to compare among treatments.

Aim 1.2. Understand the effects of silver exposure on mucous goblet cell proliferation and degeneration.

Aim 2. Understand the effects of silver expose on the fish mucus production ability.

Aim 2.1. Utilize a glucose surrogate with a phenol sulfuric acid assay to understand mucus production and mucus sloughing ability after silver exposure.

Aim 3. Identify unique biological targets of AgNP exposure.

Aim 3.1. Discern differing accumulation patterns of silver in tissue by using microwave digestion and inductively coupled plasma mass spectrometry (ICP-MS) to measure silver concentrations in a variety of tissues.

Aim 4. Characterize AgNPs in tissues of organism after uptake.

Aim 4.1. Apply flow field flow fractionation coupled to ICP-MS (FFF-ICP-MS) to establish particle form and agglomeration in tissue of exposed fish.

Aim 5. Identify differential gene expression after exposure to AgNPs or $AgNO_3$.

Aim 5.1. Perform DNA microarray analysis of the gill of FHMs to understand differential

gene expression patterns among silver treatment.

Aim 5.2. Utilize Ingenuity Pathway Analysis to understand the effects of silver treatments on toxicity pathway enrichment.

Aim 6. Understand the influence of DOC on AgNP and silver nitrate toxicity.

Aim 6.1. Understand the influence of dissolved organic carbons (DOC) on $AgNO_3$ and AgNP lethality of larval zebrafish.

Aim 6.2. Integrate data into NanoExPERT, a suite of risk assessment tools developed by collaborators at U.S. Army Corps of Engineers Engineer Research and Development Center-Environmental Lab (ERDC-EL) to improve toxicity predictions in complex water chemistries.

CHAPTER 2. GILL HISTOPATHOLOGIES FOLLOWING EXPOSURE TO NANOSILVER OR SILVER NITRATE

1. Synopsis

The fish gill is the site of many crucial physiological functions. It is among the first sites of xenobiotic exposure and gill histopathological alterations can be detected soon after toxicant exposure. Silver is one of the most toxic metals to aquatic organisms mainly due to its ability to disrupt ionic regulation. The goal of this study was to determine the effect of ionic and nanoscale silver on fathead minnow gills by examining gill histology and Na^+/K^+-ATPase immunoreactivity. Fathead minnows were exposed to two measured concentrations of $AgNO_3$ (1.3 and 3.7 µg/L as Ag^+), citrate-AgNPs (15 and 39 µg/L), and polyvinylpyrrolidone –AgNPs (11 and 50 µg/L). Circulatory disturbances were the most prevalent gill alteration detected and were statistically increased in all silver treatment groups compared to control. In addition, citrate-AgNPs significantly increased incidences of hypertrophy, lamellar fusion, epithelial lifting, and desquamation. The $AgNO_3$ (1.3 µg/L) was the only treatment that significantly increased the number of total mucous goblet cells present. In all other silver treatments, the percentage of degenerated goblet cells was statistically increased compared to control. When the sum

of all histopathological abnormalities (weighted index) was calculated, all silver groups had a significantly higher index with citrate-AgNPs having the highest toxicity (index of 10±0.32 versus 2.4±0.6 in controls). Gill Na^+/K^+-ATPase immunoreactivity was decreased by silver. These results indicated that both $AgNO_3$ and silver nanoparticles created similar disruptions in gill structure and ionic regulation possibly due to the ionic silver portion of each treatment

2. Introduction

Over 1600 consumer products are currently known to contain nanoparticles with around one-quarter of those products reporting incorporation of nanosilver (*Consumer Products Inventory* 2014). The abundant use of nanosilver in applications is largely related to natural antibacterial and antifungal properties of silver (Klasen 2000). It is included in many products such as food storage containers, toothbrushes, wound treatments, clothing and washing machines. Some of these products release silver into water (Benn et al. 2010; Benn and Westerhoff 2008); this suggests a potential exposure pathway for aquatic organisms.

Ionic silver is one of the most toxic metals to freshwater fish with relatively low LC_{50} values ranging from between 5 and 70 µg Ag/L (0.05 – 0.65 mM) when dosed as silver nitrate (reviewed by (Hogstrand and Wood 1998). Ionic silver is the most toxic form of silver to fish primarily because of disruption of gill osmoregulation (Bianchini et al. 2002; Bilberg et al. 2010). Na^+/K^+-ATPase is the primary enzyme for ionic movement and regulation in teleost gills (Varsamos et al. 2005). Ionic silver toxicity in fresh water fish is created from blockage of Na^+ and Cl^- uptake in the fish gill by inhibition of Na^+/K^+-ATPase, resulting in reduced circulation and inability to regulate fluid volume

(Hogstrand and Wood 1998; Wood et al. 1999). Cardiac failure and death ultimately occur from inhibition of brachial uptake of Na^+ and Cl^-, establishing the fish gill the prevailing site of action of silver toxicity in fish.

The fish gill is a multifunctional organ that is the site of gas exchange, ionic-regulation, acid-base regulation and excretion of nitrogenous waste (Evans et al. 2005). The morphology of the fish gill can be altered by exposure to many toxicants in the aquatic ecosystem including organophosphates (Fanta et al. 2003), copper nanoparticles (Griffitt et al. 2007), hexavalent chromium (Mishra and Mohanty 2008), carbamates and petroleum compounds (Evans 1987). Because the gill epithelium is the first site of exposure to environmental pollutants, histopathological alterations are detectable in as little as three hours following exposure to metals (Speare and Ferguson 2006). These alterations include: epithelial lifting, epithelial hypertrophy, hyperplasia, telangiectasia, circulatory disturbances, epithelial desquamation, and necrosis (Evans 1987). Many of these alterations occur due to underlying physiological changes that are created by toxicant exposure. Commonly, this results in the failure of gill cellular osmoregulation in freshwater species.

Due to the common profiles of histopathological changes after exposure to many xenobiotics, it has been noted that many of these are not toxicant-specific responses but rather indicative of a general stress response (Evans 1987; Costa et al. 2009). Gill alterations have been reported in untreated fish due to other conditions like handling stress (Costa et al. 2009). However, the pathological importance and frequency of each alteration allows for the distinction of general stress-related alterations from toxicant-related alterations (Bernet et al. 1999). In order to overcome the difficulties of the

unspecific nature of gill alterations, a weighted indices approach can be taken. One such approach considers the biological significance of each particular alteration to the fish and was proposed by Bernet and coworkers (1999) and further modified by Costa (2009). These scoring approaches allow for the conversion of histological results into numerical data and allows for statistical analysis between treatment groups. In the previously mentioned studies, the weighted indices approach was used to create cause-effect assessments for the presence of multiple toxicants in estuarine sediments which contained metals (cadmium, chromium, copper, nickel, lead and zinc) and organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dichloro-diphenyl-trichloroethane) (Costa et al. 2009) or pollution in a water system (Bernet et al. 1999).

Another critical stress response of fish gills is mucus production. Mucus aids by binding toxicants and when mucus is sloughed cellular uptake is prevented (Speare and Ferguson 2006). Because mucus can affect the pH of the gill microenvironment, it has a role in ion exchange (Varsamos et al. 2005) and/or changing the toxicity of xenobiotics (Shephard 1994) including the ionization state of metals (Handy and Eddy 1990). As exposure to an irritant increases, however, mucus can become more rigid, and the fish will not be able to slough mucus off as efficiently leading to a decreased ability of ionic regulation and the possibility of concentrating xenobiotics in the gill (Lichtenfels et al. 1996). Alternatively, if an exposure causes changes in mucous goblet cell number or viability, the capacity of the fish to produce mucus will also be adversely impacted (Pickering and Macey 1977).

Our research goal was to understand the biological responses in the fish gill, the recognized site of action of ionic silver, following AgNP exposure in the fathead minnow gill and determine if the response is unique relative to exposure to the silver ion. In this study, gill histopathological alterations such as mucous goblet cell degeneration and Na^+/K^+-ATPase activity were measured to determine whether nanosilver (specifically polyvinylpyrrolidone (PVP)- and citrate-coated nanosilver) was differentially toxic compared to $AgNO_3$. Previous studies have correlated the dissolved silver fraction from nanoparticle exposures with ionic Ag^+ concentrations revealing that the dissolved Ag^+ was the leading factor in AgNP acute toxicity (Kennedy et al. 2010; Kennedy et al. 2012). Our results showed a significant increase in the occurrence of gill alterations in fish exposed to all silver treatments, compared to controls. Citrate-AgNPs caused the highest incidence of some lesions such as epithelial desquamation. With the exception of 1.3 µg/L $AgNO_3$, all other silver treatments significantly increased the percent of degenerated mucous goblet cells and decreased the Na^+/K^+-ATPase immunoreactivity.

3. Methods

3.1 Fish source, care and handling

Fathead minnows (*Pimephales promelas*) age six to eight months (3.9-6.8 cm long) were obtained from Aquatic Bio Systems (Fort Collins, CO, USA) and cultured according to University of Mississippi IACUC approved conditions. The fish were allowed to acclimate in glass exposure chamber containing 1.5 L of moderately hard water (MHW) prepared by U.S. EPA guideline 821-R-02-013 for four days prior to the exposure. During acclimation, fish were fed with Tetramin flakes (Blacksburg, VA, USA) and water was changed daily.

3.2 Test Materials

Biopure PVP-AgNPs and citrate-AgNPs were obtained from Nano Composix (San Diego, CA, USA) at a concentration of 1 mg/mL and a nominal size of 20 nm. Concentrated stock suspensions were prepared by sonicating the stock suspension in a water bath (42 kHz, Brason Ultrasonics, Danbury, CT, USA, model 3510-DTH) for 5 minutes and inverting to mix. Concentrated stocks were diluted with nanopure water to obtain a working stock with a nominal concentration of 40 µg/mL.

3.3 Characterization and fate

Working stocks (15 mL) were characterized at the U.S. Army Engineer Research and Development Center as previously described (Kennedy et al. 2012). In order to understand particle dispersion stability in exposure medium, AgNPs were added to MHW (2 mg/L) as described above in absence of animals and particle sizing was performed at both 24 and 48 hr. Briefly, primary particle size was determined using multiple transmission microscopy images (TEM, Zeiss 10CA, 60 kV, Oberkochen, Germany) by manually measuring the longest dimension of individual particles (≥ 200) using commercially available software (ImagePro Plus, v7, Media cybernetics, Inc., Bethesda, MD). Hydrodynamic diameter (HD) was determined by both dynamic light scattering (DLS) using an intensity of light scattered autocorrelation function (635 nm laser; 90 Plus/BI-MAS, Brookhaven Instruments, Holtsville, NY) and Field Flow Fractionation (PostNova F-1000 symmetrical flow Field Flow Fractionation, St. Lake City, UT). DLS data were presented as effective diameters (ED) obtained in three sequential 3 minute durations and ranges were determined by the bounds of the log normal distribution of the EDs. The FFF particle size data were presented as the mean

of the fractogram. Silver nitrate (CAS 7761−88−8, Sigma Aldrich, St. Louis, MO) was diluted to a working stock in nanopure water with a nominal concentration of 10 µg/mL.

3.4 Exposure

Fathead minnows were exposed to control, PVP-AgNPs and citrate-AgNPs (20 and 200 µg/L, nominal) or silver nitrate ($AgNO_3$; 2 and 6 µg/L, nominal) for 96 hours (n=5 chambers/treatment; 3 fish/chamber; 1.5 L water/chamber). The nominal exposure concentrations were selected to theoretically provide equivalent dissolved Ag concentrations in the PVP-AgNPs and citrate-AgNPs treatments, when compared to the treatment levels of $AgNO_3$ based on previously reported dissolved fractions for similar AgNPs (Kennedy et al 2010, 2012). The fish were fed once at 48 hr, 30 min before water change to avoid prolonged interaction between particles and food leading to a potential dietary exposure. Water was changed and retreated daily at 9 AM. Water quality throughout the study was 307±2 µS, 218±1 mg/L TDS, 26.6±0.8℃, 149±1 mg/L salinity, and pH 8.21±0.2. The fish were euthanized with clove oil and dissected. Body weight and length were recorded and gills removed. The second gill arch from each side of each fish was placed in buffered formal saline (250 ml 40% formaldehyde, 10 g $NaH_2PO_4{\cdot}1H_2O$, 16.5 g Na_2HPO_4; diluted in DI water, pH 7.2) for 1 week at room temperature for histological analysis.

Water samples were collected at 10-30 min post initiation and 24 hr following addition of silver to confirm Ag concentration (n=5/treatment/time). To determine total Ag concentration, 10 mL of water was collected and mixed with 5 mL of 10% nitric acid and refrigerated until analysis. To determine dissolved Ag concentrations, 8 mL of water was centrifuged with an Optima Max Tabletop Ultracentrifuge (Beckman Coulter,

Fullerton, CA, USA) for 1 hr at 152,000 x *g* at room temperature (Kennedy et al. 2010; Poda et al. 2011). Following centrifugation, 4 mL of sample was carefully collected from the top to ensure that the pellet at the bottom of the tube was not disturbed. The 4 mL water sample was mixed with 2 mL of 10% nitric acid and refrigerated until analysis by ICP-MS.

3.5 Solution nebulization-ICP-MS analysis

Diluted sample solutions were analyzed for total Ag using a sector field-ICP-MS (Element XR, Thermo Fisher Scientific, USA). Sample and standard solutions were introduced via a concentric glass nebulizer coupled to a glass spray chamber. Rhodium was added online as internal standard. External calibration was performed using an Ag standard ranging from 0.1 µg/L to 1.5 µg/L (SPEX CertiPrep, USA). Silver was analyzed in low resolution mode ($m/\Delta m \approx 400$).

3.6 Gill histopathology

After fixation in buffered formal saline, the second gill arches were dehydrated by ethanol rinses and embedded in paraffin. The gills were then sectioned at 8 µm with a microtome (Olympus Cut 4055, Triangle Biomedical Sciences, Durham, NC) and stained with either hematoxylin (Fisher Scientific, Fairlawn, New Jersey) and eosin (Ricca Chemical, Arlington, Texas) to observe histopathological abnormalities or Alcian Blue-Periodic Acid-Schiff stain kit (Richard-Allan Scientific) for visualization of mucous goblet cells. One hundred secondary lamella were observed for histopathological abnormalities and 50 interlamellar spaces were observed for mucous goblet cells per each fish (n = 5). The gills were scored for abnormalities using the previously published (Costa et al. 2009; Table 2.1). The mucous cells were counted and recorded as the total

cells present and the percent of cells that had signs of degeneration as indicated by regression in size or alterations in morphology (elongation, cell wall disintegration). Gill histopathology and mucous cell degeneration was scored blind to treatment.

3.7 Gill Immunohistochemistry

All immunohistochemistry procedures were performed on paraffin sections mounted to glass histological slides. Tissue sections were deparaffinized with xylene and rehydrated with ethanol (100, 95, 75, and 50%). Antigen retrieval were performed by immersing slides with bound tissue in boiling 10 mM sodium citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 min. Slides were then allowed to cool on the bench top for 30 min, fixed with 4% formaldehyde and washed with 1X PBS 2 times for 5 min. Then slides were incubated with 0.3% Triton X-100 in PBS for 5 min and washed 2 times for 5 min in 1X PBS with 1% bovine serum albumin (BSA). Nonspecific binding was blocked with blocking buffer (Life Technology) for 1 hr and followed by overnight incubation at 4℃ with monoclonal primary antibody for Na^+/K^+-ATPase (mouse anti-Na^+/K^+-ATPase α-subunit from chicken (1:250;antibody α5; Iowa Hybridoma Bank, University of Iowa, IA) in blocking buffer. Then a FITC- goat anti-mouse IgG (Invitrogen) was used to identify Na^+/K^+-ATPase and incubated for 1 hr. Slides were washed twice with 1X PBS, covered with anti-fading Prolong mounting medium (Invitrogen), mounted, and analyzed by epifluorescent microscopy (Zeiss Axioskop 2 upright microscope fitted with bright field and epifluorescence illumination and Zeiss Axiocam digital imaging system for documentation, using Improvision's Openlab imaging software for image capture). Negative controls were performed by replacing the primary antibody with blocking buffer.

3.8 Statistics

Results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean±S.E. Data sets were first analyzed for normality by the Kolmogorov-Smirnov test. If data passed normality, one-way ANOVA followed by Newman-Keul's post hoc test was used. If data was not normally distributed, Kruskal-Wallis followed by Dunn's post hoc test was used. Statistical significance was accepted at $p \leq 0.05$ for all tests.

4. Results

4.1 Particle characterization and water Ag concentrations

The measured diameters for PVP-AgNPs (22 ± 2 nm) and citrate-AgNPs (21 ± 4 nm) determined from TEM images closely agreed with the nominal size of 20 nm, while measurement of hydrodynamic diameter was slightly larger (Figure 2.1 A,B). Based on the hydrodynamic diameter, citrate-AgNPs agglomerated within 24 hr in MHW while PVP-AgNPs dispersions were relatively more stable (Figure 2.1 C-E).

Measured total Ag water concentrations averaged 1.3 ± 0.05 and 3.7 ± 0.16 µg/L for $AgNO_3$, 15 ± 1.1 and 39 ± 3.6 µg/L for citrate-AgNPs, and 11 ± 0.74 and 50 ± 4.2 µg/L for polyvinylpyrrolidone –AgNPs (Figure 2.2A). The Ag concentrations remained constant throughout the exposure (as indicated by small standard error bars on Fig. 2.2). The concentrations were also comparable at both the 30 min and 24 hr after silver addition (Figure. 2.2A,B). The actual concentrations of both doses of $AgNO_3$ and the low doses of PVP-AgNPs and citrate AgNPs were 55-75% of the nominal concentrations, while the high dose of PVP-AgNPs and citrate-AgNPs were 20-25% of

the target concentrations (Figure 2.2A). Both low and high doses of PVP-AgNPs and citrate-AgNPs resulted in an average of 2% and 1.7% dissolved Ag, respectively (Figure 2.2). The dissolved concentrations were significantly different between low (0.2-0.3 µg/L) and high (0.8-1.1 µg/L) dose groups but did not vary significantly across treatments within exposure level.

A

Analysis	NC20 Citrate (nm)	NC20 PVP (nm)
Nominal	20	20
Primary particle (ERDC/NanoSafe TEM)	21 ± 4 (3-146)	22 ± 2 (10-56)
Hydrodynamic diameter (ERDC – FFF)	29	29
Hydrodynamic diameter (ERDC – DLS)	29.5 ± 0.1	34.8 ± 0.4

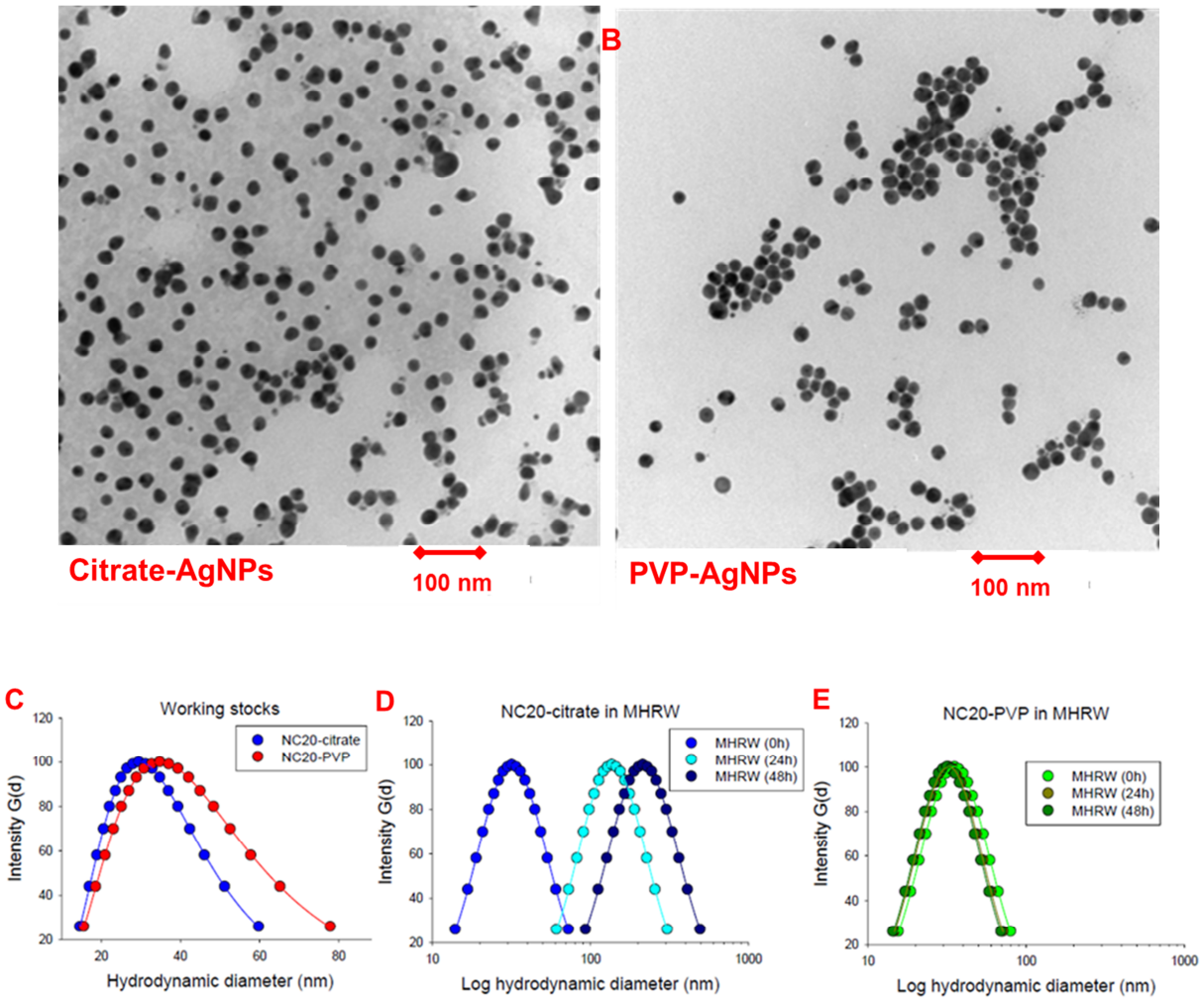

Figure 2.1. Silver nanoparticle characterization. A) Summary table of particle size in nm properties. B) TEM images of citrate-AgNPs and PVP-AgNPs, respectively. C-E) Stability of nanoparticles in MHW over 48 hr.

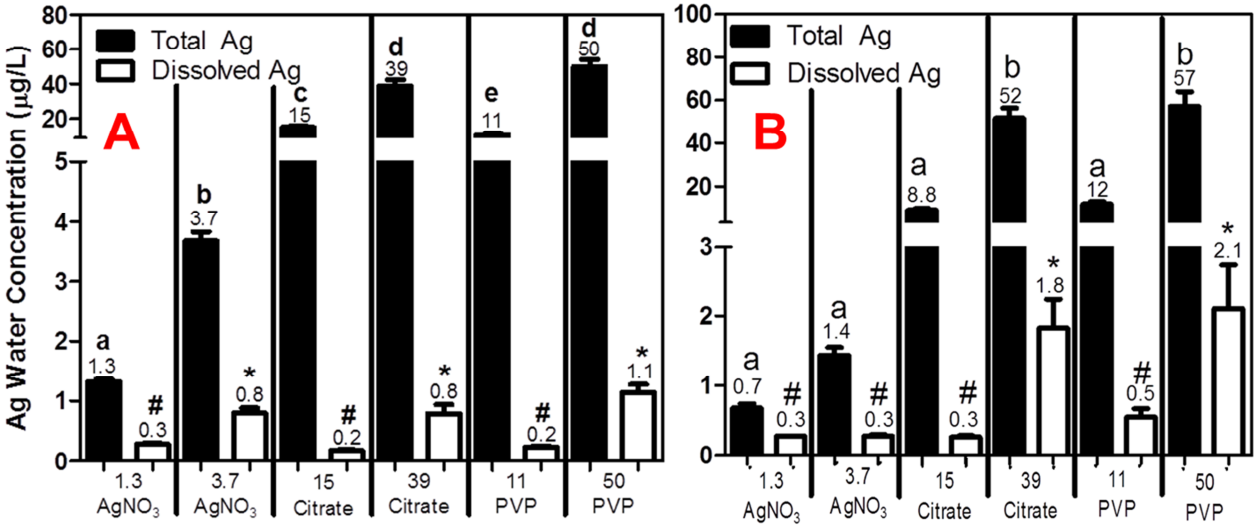

Figure 2.2 Ag concentrations in FHM water samples. Total and dissolved Ag concentrations from water samples taken at ~30 min (A) or 24 hr (B) after dosing during fathead minnow. Concentrations were log transformed and then ANOVAs with Tukey's post-hoc test was used for analysis ($p<0.05$; n=11 samples per treatment per time). Comparisons among total Ag were analyzed (letters) and comparisons among dissolved Ag were analyzed (symbols). Bars with different letters/symbols are significantly different.

4.2 Gill histopathology

A gill arch from one fish per each of the five chambers per treatment were scored (n=5; Figure 2.3). All treatment groups, including controls, displayed some histopathological alterations with circulatory disturbances being the most common alteration observed. In order to account for background handling stress, the average number of occurrences of each alteration in the control fish was measured, and occurrences of alterations in the dosed fish were assigned a weighted score only if the total occurrences for exposed animals were higher than the average number of occurrences in control fish. Alterations were scored with either a one or two depending

on the pathological importance of the alteration (Table 2.1). Following the Costa approach, mucous goblet cell degeneration and epithelial hypertrophy were weighted higher due to an increased pathological importance and decreased ability for alteration reversal. The other alterations were weighted with a one because they are not as pathologically important and more easily reversed after exposure to the stressor ends.

Table 2.1. Gill histopathological alterations. Percent incidence (± SEM) of gill histopathological alterations present following Ag exposure. Gills were scored blind to treatment following hematoxylin and eosin staining and 100 lamellae per fish were scored. Percentages of each alteration were calculated per fish and averaged per treatment group (n=5 fish/treatment). The *condition weight (1 or 2) for each alteration was based from Costa (2009). Data within a column with different letters are statistically different.

	Reaction Patterns								
	Circulatory			Regressive			Progressive		
	Telangiectasia	Circulatory Disturbances	Epithelial Lifting	Epithelial Desquamation	Deformed Lamellae	Mucus Cell Degeneration	Epithelial Hypertrophy	Lamellar Fusion	Epithelial Hyperplasia
Treatment	1*	1*	1*	1*	1*	2*	1*	1*	2*
Control	0^{a}	47 ± 2.7^{a}	6.6 ± 2.5^{a}	1.6 ± 0.7^{a}	0^{a}	7.3 ± 3.2^{a}	22 ± 3.1^{a}	0^{a}	0^{a}
1.3 $AgNO_3$	0^{a}	85 ± 3.3^{b}	33 ± 4.9^{ab}	22 ± 8.6^{ab}	3.4 ± 1.9^{a}	28 ± 6.1^{a}	50 ± 10^{ab}	3.0 ± 1.2^{ab}	0.2 ± 0.2^{a}
3.7 $AgNO_3$	2.0 ± 1.6^{a}	90 ± 3.9^{bc}	52 ± 10^{b}	25 ± 9.2^{ab}	6.2 ± 3.7^{a}	61 ± 9.8^{b}	35 ± 7.4^{ab}	3.8 ± 1.7^{ab}	4.8 ± 2.9^{a}
11 PVP	0^{a}	93 ± 4.1^{bc}	29 ± 4.3^{ab}	37 ± 9.1^{ab}	12 ± 10^{a}	67 ± 11^{b}	55 ± 11^{ab}	2.2 ± 1.1^{ab}	2.4 ± 1.1^{a}
50 PVP	0^{a}	85 ± 2.5^{bc}	42 ± 9.1^{ab}	7.2 ± 2.7^{a}	2.8 ± 2.3^{a}	57 ± 11^{b}	29 ± 7.5^{ab}	0^{a}	0^{a}
15 Citrate	0.8 ± 0.6^{a}	98 ± 1.3^{c}	32 ± 5.4^{ab}	47 ± 9.0^{b}	19 ± 7.6^{a}	60 ± 7.4^{b}	66 ± 8.3^{b}	4.6 ± 1.1^{b}	6.6 ± 2.1^{a}
39 Citrate	0.6 ± 0.6^{a}	95 ± 2.4^{bc}	48 ± 11^{b}	27 ± 12^{ab}	21 ± 10^{a}	66 ± 8.2^{b}	54 ± 11^{ab}	1.2 ± 0.4^{ab}	6.0 ± 1.5^{a}

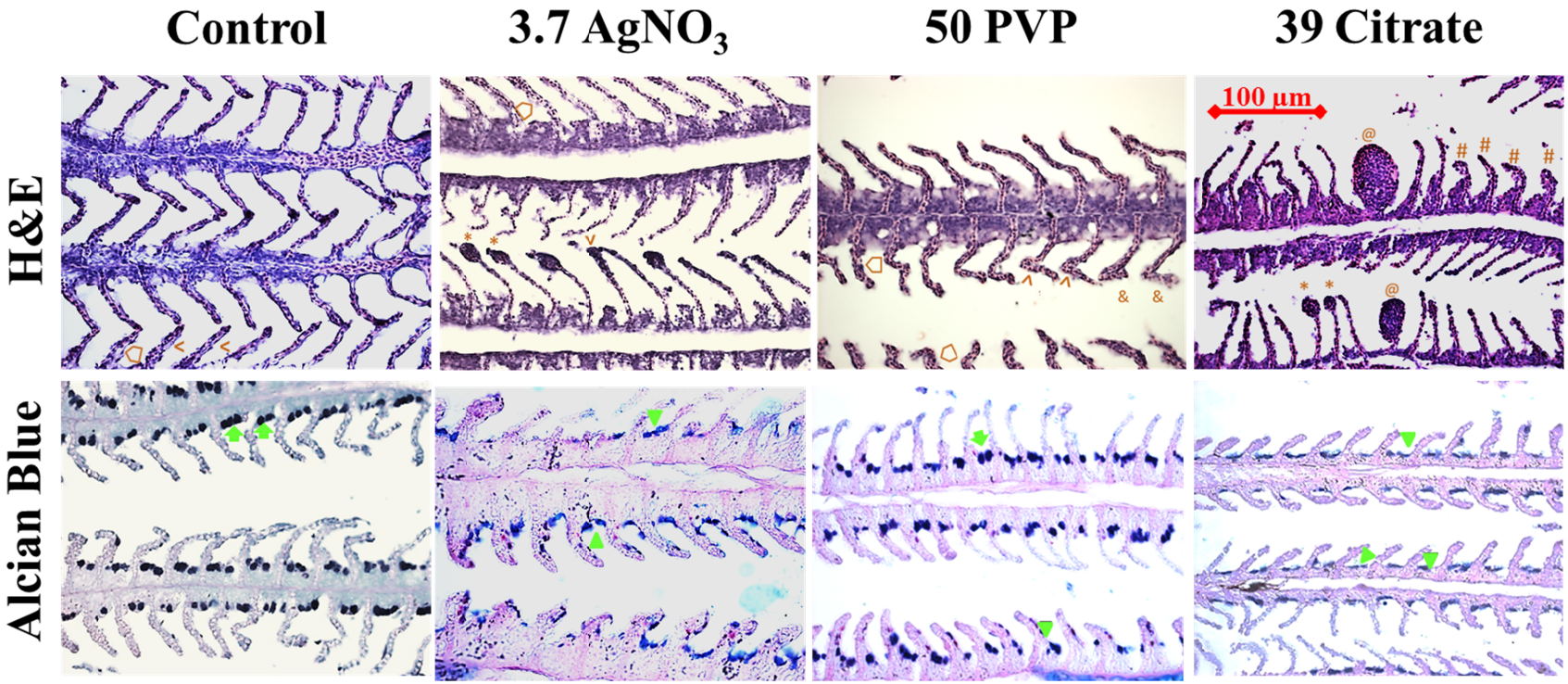

Figure 2.3. Gill histopathological images: Hematoxylin and eosin or alcian blue-periodic acid-schiff stained gill sections from control, 3.7 µg/L $AgNO_3$, 50 µg/L PVP-AgNPs, or 39 µg/L citrate-AgNPs. Photos taken at 40x with Axioskop 2 upright microscope with Axiocam using Openlab imaging software. Specific example lesions are indicated as follows in H&E stained slides (top row): telangiectasia (@), circulatory disturbances (**<**), epithelial lifting (⌂), epithelial desquamation (#), deformed lamellae (&), and epithelial hyperplasia (*) present. Both normal mucous goblet cells (arrow) or degenerated mucous goblet cells (arrow head) are indicated in the alcian blue-PAS stained sections (bottom row).

The total number of mucous goblet cells was similar among all treatment groups (96 per 50 inter-lamellar regions) except that the low $AgNO_3$ had significantly more than control (1.9 – fold) (Figure 2.4). However, in both $AgNO_3$ and PVP-AgNPs treated fish, there was a trend wherein the lower Ag concentrations had higher numbers of mucous cells than fish from the high exposures. All treatment groups, with the exception of 1.3 µg/L $AgNO_3$, showed significantly greater degeneration of mucous goblet cells compared to controls (Table 2.1), which would reduce the ability of treated fish to produce mucus.

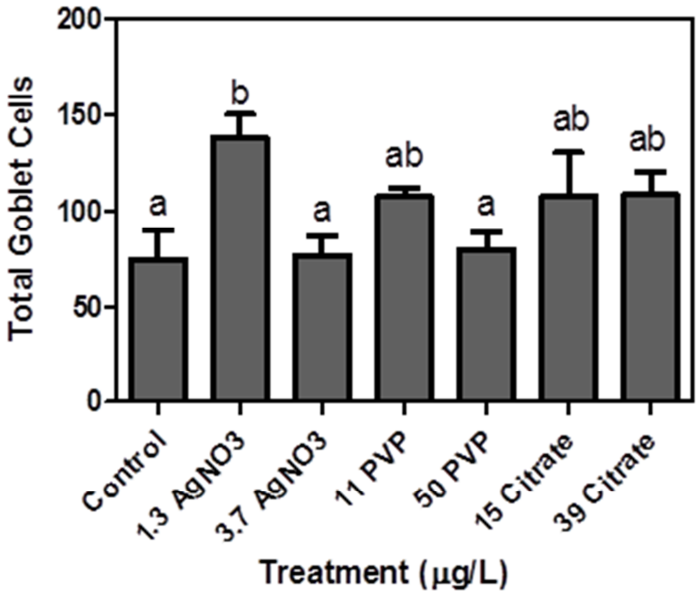

Figure2.4. Total mucous goblet cells. The sum of mucous goblet cells in 50 inter-lamellar non-lesioned areas as determined from alcian blue staining. Bars with different letters are significantly different from each other (ANOVA, SNK post-hoc, $P<0.05$, n=5 fish per treatment).

After assignment of scores based on presence and physiological importance of each gill alteration, a total index score was calculated by the sum of all present alterations in each gill. On average, fish from every Ag treatment had significantly

higher histopathological alteration indexes compared to controls (Figure 2.5). Citrate-AgNPs (15 and 39 µg/L) had the highest incidence of alterations (4.2 and 4.1-fold higher than control, respectively). Fish exposed to $AgNO_3$ displayed a dose-dependent increase in the weighted index, whereas the high dose PVP-AgNP exposed animals had significantly fewer lesions as expressed by the weighted index than the low dose PVP-AgNPs (Figure 2.5). Overall, the citrate-AgNP exposed fish had no significant dose-dependent response intensity by the weighted index approach but individual lesions were significantly higher at the lower citrate dose for circulatory disturbances, hypertrophy, lamellar fusion, and desquamation (Table 2.1). Some lesions including telangiectasia, deformed lamellae, and epithelial hyperplasia, were intermittently present but never occurred consistently more frequently in any particular treatment group.

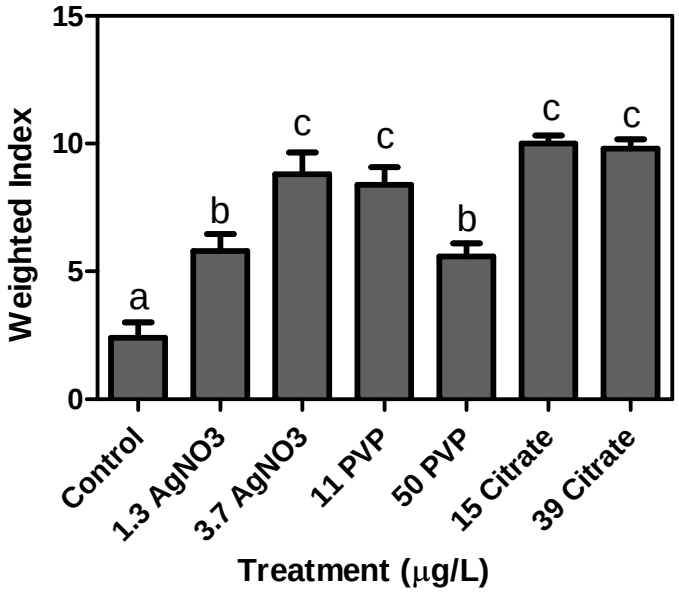

Figure 2.5. Total weighted index of gill histopathology. Weighted index represents the sum of all histopathological abnormalities, including percent degenerated mucous cells (greater than 50% degenerated were assigned a score of 2), in the gill as scored according to the matrix in Table 1. Bars with different letters are significantly different from each other (ANOVA, SNK post-hoc, $P<0.05$; n=5).

4.3 Gill immunohistochemistry

Control fish gills displayed the greatest immunoreactivity of Na^+/K^+-ATPase as indicated by the number of green fluorescent punctuate spots in Figure 2.6. Animals exposed to both $AgNO_3$ and AgNPs were determined to have decreased Na^+/K^+-ATPase immunoreactivity by treatment blinded observation of reduced fluorescence. Fish exposed to the lower concentrations in all treatments still displayed some Na^+/K^+-ATPase immunoreactivity. Na^+/K^+-ATPase was located in both the interlamellar space and in the lamellae of the fish.

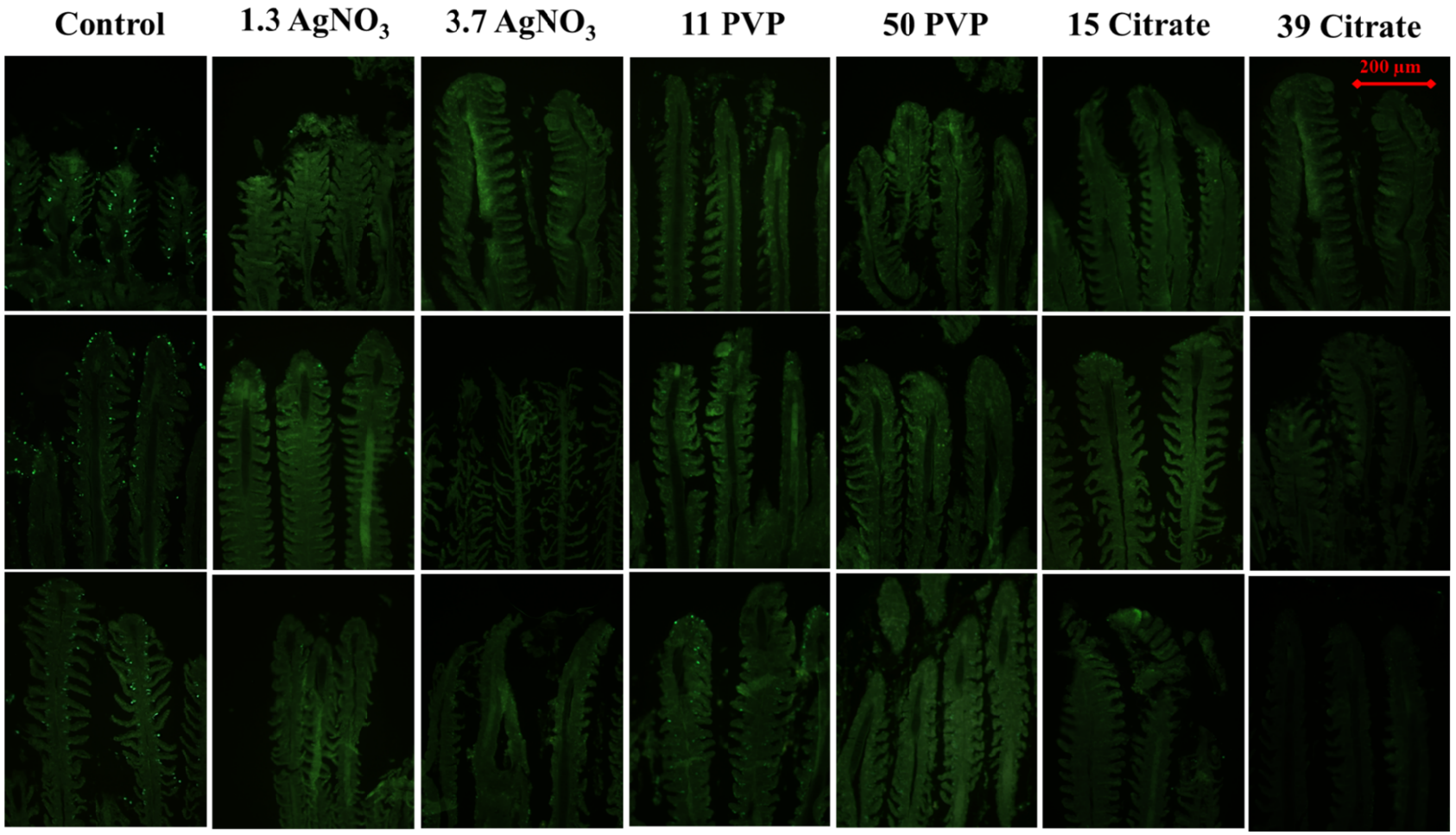

Figure2.6. Epifluorescent images of the presence of Na^+/K^+-ATPase in gills. Images captured with a Zeiss Axioskop 2 epifluorescence illumination (FITC filter set) utilizing a Zeiss Axiocam with Openlab software. Immunoreactivity indicated by bright green fluorescence punctuate spots (tissue background still present). Three of five replicates for each treatment shown.

5. Discussion

In the present study, total silver concentrations were lower than the expected nominal dose in all silver treatments. The reduction in expected concentrations has been reported by others (Newton et al. 2013; Griffitt et al. 2009; Scown et al. 2010) and could be due to silver adhering to vessel walls, heteroaggregation with biological medium such as mucus upon introduction to the exposure chamber, or the high sample dilution requirements for the ICP-MS. Exposure concentrations were chosen so that they would be environmentally plausible, sub-lethal to fathead minnows, and would yield similar ionic silver concentrations within treatment levels. While the measured amount of total silver present was less than expected, there were non-significant differences in ionic Ag^+ concentrations in all low or high dose treatments.

All fish that were exposed to silver of any formulation had a significantly higher gill alteration weighted index compared to controls. While AgNP-dosed fish were exposed to approximately 10-fold more total silver, they did not have a correspondingly higher incidence of gill histopathological alterations. While not significantly greater in the overall index of gill alterations, citrate-AgNPs yielded a greater response than the other treatment groups. Citrate-AgNPs caused increased incidences of lamellar fusion, epithelial lifting, and circulatory disturbances, possibly due to release of more Ag^+ ions because the citrate-AgNPs are less stable over time. Because ionic Ag^+ concentrations were not different within treatment levels and most of the lesions detected were common among fish exposed to $AgNO_3$ and AgNPs, the resultant gill histological effects are thought to be due to the dissolved silver fraction. The bioavailability of silver ions is thought to be the major driver of toxicity in aquatic organisms exposed to AgNPs

(Newton et al. 2013; van Aerle et al. 2013; Zhao and Wang 2012). Toxicity generated from silver ions released from AgNPs has been previously observed in a number of aquatic organisms. For example, zebrafish (*Danio rerio*) had an EC_{50} of 160.5 µg/L when exposed to PVP-AgNPs but when exposed to AgNPs with dispersant, the EC_{50} was lowered to 87.7 µg/L which was similar to the EC_{50} of $AgNO_3$ (78.11 µg/L) due to similar concentrations of Ag^+ ions (Wang et al. 2012). In Japanese medaka, aged AgNPs were more lethal (LC_{50} = 1.44 mg/L) than fresh AgNPs (LC_{50} = 3.53 mg/L), which was confirmed to be due to the release of silver ions by kinetic analysis (Bilberg et al. 2012; Kim et al. 2013; Kennedy et al. 2012).

Other studies, however, have indicated unique effects generated by AgNP exposure when different sublethal assessment endpoints are considered. In zebrafish exposed to both AgNPs and ionic forms, the gill tissue burden was greater after AgNPs exposure while the ionic silver treatments resulted in a greater thickening of gill lamellae. Furthermore, microarray analysis indicated differential gene expression between the forms of silver studied (Griffitt et al. 2009). Garcia-Reyero et al. (2014) reported both similarities and differences in *P. promelas* gene expression patterns in exposures to silver ions versus PVP-AgNPs, depending on the gene and pathway. For instance, there were commonalities in gene expression patterns between ions and particles for ion exchange homeostasis and oxidative stress but differences suggested for neurotoxicity pathways. The similarities and differences in toxic phenotypes resulting from nanometal exposures were reviewed by Shaw and Handy (2011). It is important to note that there could be distinctive pathways and biological targets that could result in lethality following longer term AgNP exposure. Differing toxicities could

be a result of different exposure kinetics due to the possibility that nanoparticles more slowly release ionic Ag causing more chronic effects (Schluesener and Schluesener 2013; Volker et al. 2013).

A gill histopathological index approach was proposed as a way to better understand and differentiate the mechanisms of toxicity of diverse types of xenobiotics. For example, after *Jenynsia multidentata* were exposed to glyphosate herbicide Roundup®, a prevalence of circulatory disturbances in controls and significantly higher progressive and regressive changes in gill histology of exposed fish were detected (Hued et al. 2012). In another study, *Epinephelus chlorostigma* gills were analyzed after exposure to light Arabian crude oil, dispersant, or dispersed oil and similar lesions were found in all treatment groups (Agamy 2013) and to those caused by silver exposure in our study. These studies suggest that while fish gill histopathological indices are useful in understanding the dose-response relationships in severity and long term heath implications due the irreversibility of some types of lesions, indices and particular lesion types are not easily assigned to a specific toxicant.

It is also important to note that gill histopathological analysis for use as a routine toxicological biomarker is not without its disadvantages. Fixing and sectioning fish gills because of their unique three dimensional structure is challenging and the extensive lesion scoring required for robust statistical comparisons in index results is time intensive. Yet, these methodological disadvantages are offset by the higher pathological relevance of phenotypic analyses that can be achieved for each fish. In contrast, gene expression changes that can be measured by PCR more quickly may be harder to link to direct adverse health outcomes.

Prior to this study, attempts to understand how AgNP exposure affects the presence and degradation of mucus producing goblet cells have not been published. The decreased ability of mucous goblet cells to secret mucus is pathologically important because mucus can act as a protective barrier to xenobiotic exposure (Speare and Ferguson 2006) and degenerated mucous goblet cells will atrophy and detach leading to a reduction in the ability to secret mucus (Barja-Fernandez et al. 2013; Monteiro et al. 2009; Salamat and Zarie 2012). Certain stressors including nanosilver (Chapter 3), metals (McDonald and Wood 1993), TiO_2 nanoparticles (Federici et al. 2007) and carbon nanotubes (Smith et al. 2007) cause mucus hypersecretion in freshwater fish. In order to understand the relationship of mucous goblet cells and silver exposure, the total number of mucous goblet cells and the number showing signs of degeneration were counted. The total mucous goblet cell counts showed a trend where there were ~100 cells per 50 interlamellar spaces in the lower concentrations of all treatment groups as compared to 75 cells in controls. The higher number of mucous goblet cells in fish exposed to the lower concentrations of silver suggests a progressive response to facilitate the production of more mucus in response to the silver stress. In contrast, in tissues from all of the higher doses of silver there were fewer cells (Figure 2.4) compared to the corresponding lower dose treatment and a higher percentage of degenerated cells (Table 2.1); a more regressive response. In fact, other work with FHMs in our lab has shown that following exposure to comparable concentrations of AgNPs, in the first 24 hr of exposure mucus hypersecretion was indicated presumably as a protective response mechanism, but by 96 hr mucus production by Ag treated fish was not significantly different than controls (Chapter 3). All the histology in this study

was conducted after 96 hr of exposure, but future work could better pinpoint the time-course associated with the transition between mucus–dependent protection and mucous goblet cell toxicity.

As expected, the presence of gill Na^+/K^+-ATPase was depleted by exposure to silver. When rainbow trout (*Oncorhynchus mykiss*) were exposed to similar concentrations of $AgNO_3$ (2 and 10 µg/L), brachial Na^+ and Cl^- influxes were almost completely inhibited at 8 hr after exposure (Morgan et al. 1997). In a similar experiment, Na^+/K^+-ATPase enzyme activity was significantly reduced at 24 hr post silver exposure in *O. mykiss* (Morgan et al. 2004). While depletion of brachial Na^+ and Cl^- influxes cannot be totally attributed to reduction of Na^+/K^+-ATPase (Morgan et al. 2004), it is the major enzyme controlling osmoregulation in fish and disruption of function can lead to reduced ionic regulation, morphological changes, cardiac arrest and ultimately death of the animal (Speare and Ferguson 2006; Hogstrand and Wood 1998). The conserved responses in the FHMs from the different silver treatment groups including similar histopathologies, mucous goblet cell degeneration, and Na^+/K^+-ATPase depletion leads to a conclusion that the acute toxicity to FHM gills was likely derived from the ionic fraction of silver even when exposed to AgNPs. However, it is not possible to rule out other areas where AgNPs could still generate unique particle effects especially considering potential extended time of ionic exposure due to slow release ions, unique routes of exposure, and the possibility of AgNPs reaching different biological targets.

The results from this study show that using gill histopathology as the endpoint, fathead minnows respond to AgNPs and $AgNO_3$ in a similar fashion after 96 hr

exposures. The compromised ability to osmoregulate and respiratory stress would eventually lead to death, making increasing concentrations of AgNPs in aquatic ecosystems worthy of further consideration in risk assessments. While some of the concentrations used in this exposure were higher than current amounts of silver known in the aquatic environment, these concentrations could realistically be met at the point source of pollution or if the usage of AgNPs continues to increase at the current rate.

6. Acknowledgements

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CHAPTER 3. ALTERATION IN *PIMEPHALES PROMELAS* MUCUS PRODUCTION AFTER EXPOSURE TO NANOSILVER OR SILVER NITRATE

1. Synopsis

The fish gill's ability to effectively produce mucus is a critical part of the stress response and protection against xenobiotic toxicity. Adult fathead minnows were exposed to $AgNO_3$ (0.82 or 13.2 µg/L), polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs) (11.1 or 208 µg/L) and citrate-coated AgNPs (10.1 or 175 µg/L) for 96 hr. Mucus concentrations based on glucose as a surrogate were determined at 0, 1, 2, 3, 4 and 24 hour after re-dosing each day. Higher mucus production rates were observed at the beginning as compared to after 3 days of exposure and by silver-treated fish. Control fish produced consistent mucus concentrations throughout exposure (0.62 and 0.40 mg/L at 24 and 96 hours, respectively). Following 24 hr of exposure, all silver treatment groups produced significantly more mucus than controls. Following 96 hr of exposure, mucus concentrations in treatment groups were significantly reduced compared to each respective treatment at 24 hr. Reduced mucus production following long term silver exposure could prevent the gills from removing silver, and thus increase toxicity.

2. Introduction

The antimicrobial properties of nanosilver have led to its increasing use in commercial products (e.g. food storage containers, textiles, athletic equipment, and medical devices). Some of these products release silver into waste water (Benn et al. 2010; Benn and Westerhoff 2008) creating an environmental concern because of the known toxicity of ionic silver to aquatic organisms. Further complicating environmental risk assessment is the potential for unique toxic effects and biological targets of AgNPs as compared to dissolved ionic Ag (Fabrega et al. 2011).

Mucus is essential for protection from xenobiotic exposure in fish because it forms a physical barrier to protect tissues including the gill, skin, and/or intestine from the surrounding environment (Shephard 1994). Mucus hypersecretion is a common stress response to toxicants and irritants (Mallatt 1985). Hypersecretion of mucus is protective by both trapping and sloughing xenobiotics, but also when innate immune proteins such as lysozyme and IgM are introduced to pathogens via mucus (Magnadóttir 2006). Mucus also absorbs H^+ ions and, thus, plays a role in ionoregulation by buffering substances introduced to the gill microenvironment (Handy et al. 1989; Handy and Eddy 1991).

Fish exposed to metals (McDonald and Wood 1993), carbon nanotubes (Smith et al. 2007), and TiO_2 nanoparticles (Federici et al. 2007) have displayed mucus hypersecretion. While mucus production can help reduce metal toxicity as described above (Coello and Khan 1996; Handy 1992), there are also concerns about mucus enhancing contaminant toxicity. If the mucus layer becomes more rigid after stressor exposure, it will not be sloughed and then contaminants such as AgNPs can be

concentrated at the gill surface and persistently release damaging Ag^+ ions (Lichtenfels et al. 1996). The presence of a thicker layer of mucus also reduces the gas exchange, ionic regulation, and nitrogenous waste excretion functions of the fish gill. Hypersecretion of mucus can cause the loss of goblet cells which, in turn, diminishes an organism's ability to secrete mucus during subsequent exposures (Mallatt 1985). Thus, the present study aimed to understand if the ability of fathead minnows (*Pimephales promelas*) to produce mucus was altered over the course of a 96 hr exposure to silver nitrate ($AgNO_3$), citrate-coated (citrate-AgNPs), or polyvinylpyrrolidone-coated (PVP-AgNPs) silver nanoparticles.

3. Methods and Materials

3.1 Fish source, care and handling

Six to eight month old (3.4 to 6.6 cm) fathead minnows (FHMs) (*Pimephales promelas*) were obtained from Aquatic Bio Systems (Fort Collins, CO, USA) and acclimated in glass exposure chambers for four days containing 1.5 L of moderately hard water (MHW) prepared by U.S. EPA guideline 821-R-02-013 under University of Mississippi IACUC approved conditions. During acclimation, fish were fed Tetramin flakes (Blacksburg, VA, USA) and water was changed daily.

3.2 Silver

PVP-AgNPs and citrate-AgNPs were obtained from Nano Composix (San Diego, CA, USA) at a concentration of 1 mg/mL and a nominal size of 20 nm. Concentrated stocks were sonicated in a bath sonicator for 5 min and inverted to mix. Concentrated

stocks were diluted with nanopure water to obtain a working stock with a nominal concentration of 40 µg/mL. The mean of the field flow fractionation (PostNova F-1000 symmetrical flow FFF, St. Lake City, UT) fractograms confirmed particle sizes of 26 and 27 nm for PVP-AgNPs and citrate-AgNPs, respectively. Silver nitrate was obtained from a commercial source (CAS 7761−88−8, Sigma Aldrich, St. Louis, MO) and diluted to a working stock in nanopure water to a nominal concentration of 10 µg/mL.

3.3 Exposure

Fathead minnows were exposed to control, PVP-AgNPs (measured concentrations: 11.1±1.6 or 208±40.7 µg/L), citrate-AgNPs (10.1±0.6 or 175±5.2 µg/L) or silver nitrate ($AgNO_3$; 0.82±0.2 or 13.2±1.6 µg/L) for 96 hours (n=5 chambers/treatment; 3 fish/chamber; 1.5 L water/chamber). The doses were chosen to provide a similar dissolved Ag concentration in the AgNPs and the $AgNO_3$ treatments. Water was changed, chambers rinsed, and redosed every 24 hr. The fish were fed once at 48 hr, 30 min before the water change at 9 AM. Water parameters were 289±2 µS, 231±1 ppm TDS, 26.4±0.8℃, 151±1 ppm salinity, and pH 8.09±0.3. Water samples were taken immediately following dosing to confirm total Ag concentration (n=5/treatment/collection). To determine total Ag concentration, 10 mL of water were collected and mixed with 5 mL 10% nitric acid and refrigerated until analysis. Diluted sample solutions were then analyzed for Ag using a sector field-ICP-MS (Element XR, Thermo Fisher Scientific, USA). At 96 hr, the fish were euthanized with buffered MS-222. Body weight and length were recorded.

3.4 Mucus water concentrations

The estimation of mucus water concentration was done by using the phenol sulfuric acid assay protocol established by (Dubois et al. 1956) and modified for this application by (Parrish and Kroen 1988). Ten mL of water from each chamber (n=5) were collected at each time point (0, 1, 2, 3, 4 and 24 hr post dosing throughout the exposure starting at 0, 24, 48, 72 and 96 hrs) and lyophilized for ~48 hr. The samples were then reconstituted into 50 µL of nanopure water and diluted to 2.5 mL with 1:1.5 v/v 20% phenol:sulfuric acid. Of this mixture, 250 µL was pipetted in triplicate into a 96 well plate and measured at 492 nm on a Perkin Elmer HTS7000 plate reader. Glucose was used as a standard curve to represent the mucus carbohydrate content. The glucose standard curves were linear between 0.004 and 0.5 mg/mL with an average r^2 of 0.977.

3.5 Statistics

Results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean±S.E. Data sets were first analyzed for normality by the Kolmogorov-Smirnov test. If data passed normality, one-way ANOVA followed by Tukey's post hoc test was used. If data was not normally distributed, Kruskal-Wallis followed by Dunn's post hoc test was used. Statistical significance was accepted at $p \leq 0.05$ for all tests. Mucus concentration vs. time was plotted in Graphpad Prism 5 to obtain the mucus production rate and calculate the correlation by using linear regression analysis.

4. Results

The mucus production rates for exposure hours 1 to 4 and exposure hours 73 to

77 are shown in Figure 3.1 with corresponding slopes and r^2 values indicated in Table 3.1. Initial and final mucus production rates (0.088±0.007 and 0.101±0.029 mg/L/hr, respectively) were similar in control fish (Figure 3.1A). Fish from all treatment groups had higher rates of mucus production and higher r^2 correlation coefficients in the initial 4 hours of the exposure compared to three days into the exposure (e.g. between 73 and 76 hrs) (Figure 3.1B-D). Initially each of the higher silver concentrations (e.g. 13 µg/L $AgNO_3$, 208 µg/L PVP-AgNPs, and 175 µg/L citrate-AgNPs) also had higher mucus production rates compared to the lower concentrations of silver. The highest rate (0.779 mg/L/hr) was found in fish from the 13 µg/L $AgNO_3$ treatment group. By day three of the exposure, all Ag treatments seemed to have greatly reduced ability to produce mucus. For example, the mucus production rates in the high dose PVP-AgNP and citrate-AgNP groups were only 26 and 29%, respectively of the rate measured in hours 1 to 4 of the exposure.

At 24 hours post silver exposure, every Ag treatment produced a greater amount of mucus than the controls (Figure 3.2). There was a statistically significant dose-dependent increase in mucus secretion at 24 hrs in each silver treatment. By 96 hr, control fish were still producing a consistent amount of mucus (not significantly different from controls at 24 hr post dosing). However, by 96 hr, the ability to produce mucus in all but the low dose citrate-AgNPs was not statistically different than controls indicating inability to respond to sustained Ag stress after four days of treatment. In fact, by as early as 48 hr (data not shown) sloughed mucus concentrations were not statistically different between control and all silver treatments except the 0.82 µg/L $AgNO_3$.

Table 3.1. Mucus production rate and correlation efficient following 1 -4 and 73-76 hours of exposure. Rates were calculated by plotting mucus concentration vs. time (1, 2, 3, and 4 hr) on day 0 and 3 of exposure (n=5/treatment/time point).

	Hours 1-4		Hours 73-76	
Treatment (µg/L)	**Slope ± SE**	**r^2**	**Slope ± SE**	**r^2**
Control	0.088±0.007	0.899	0.101±0.029	0.407
0.82 $AgNO_3$	0.581±0.079	0.753	0.243±0.044	0.623
13 $AgNO_3$	0.779±0.051	0.928	0.236±0.040	0.655
11 PVP-AgNPs	0.495±0.027	0.950	0.261±0.100	0.276
208 PVP-AgNPs	0.743±0.042	0.945	0.196±0.044	0.528
10 Citrate-AgNPs	0.482±0.032	0.926	0.304±0.081	0.439
175 Citrate-AgNPs	0.716±0.047	0.928	0.211±0.051	0.483

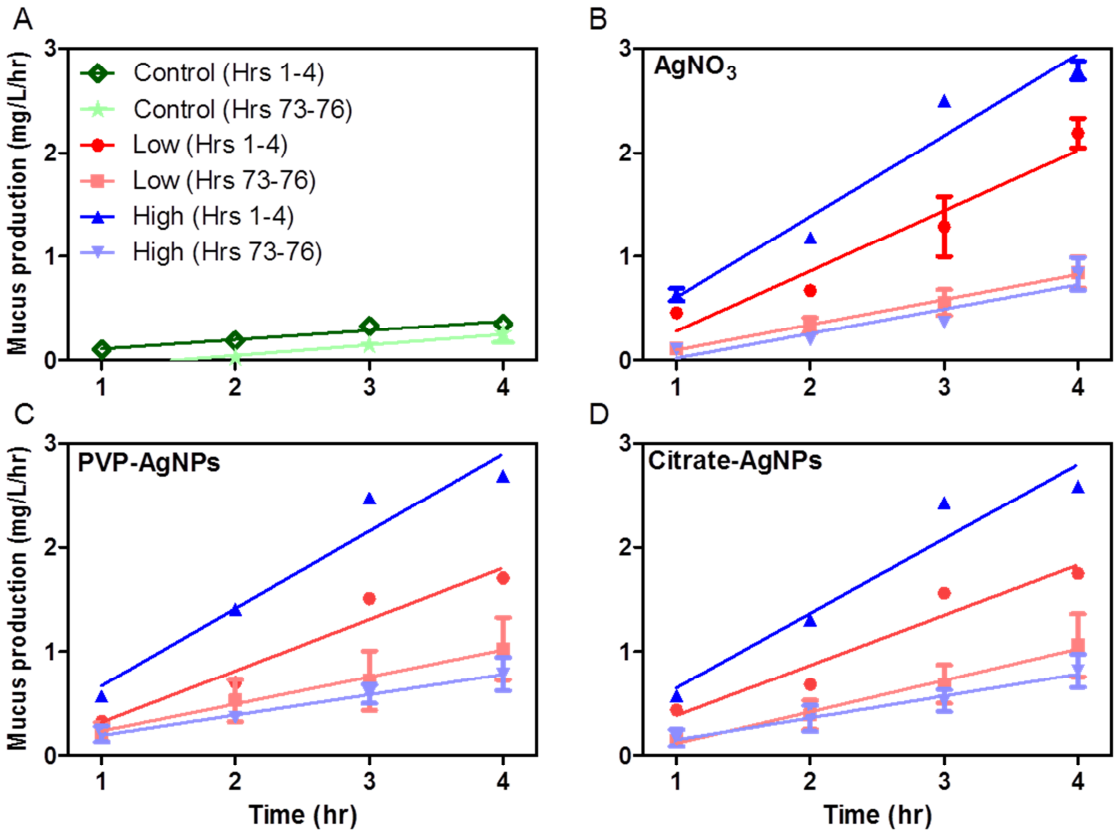


Figure 3.1. Mucus production rate during hours 1 to 4 and 73 to 76 following exposure to control, low and high concentrations of silver. Control (A), 0.82 and 13.2 µg/L $AgNO_3$ (B), 11.1 and 208 µg/L PVP-AgNPs (C), and 10.1 and 175 µg/L citrate-AgNPs (D). Linear regression analysis within treatment and day was used to determine the rate (mg/L/hr) and correlation.

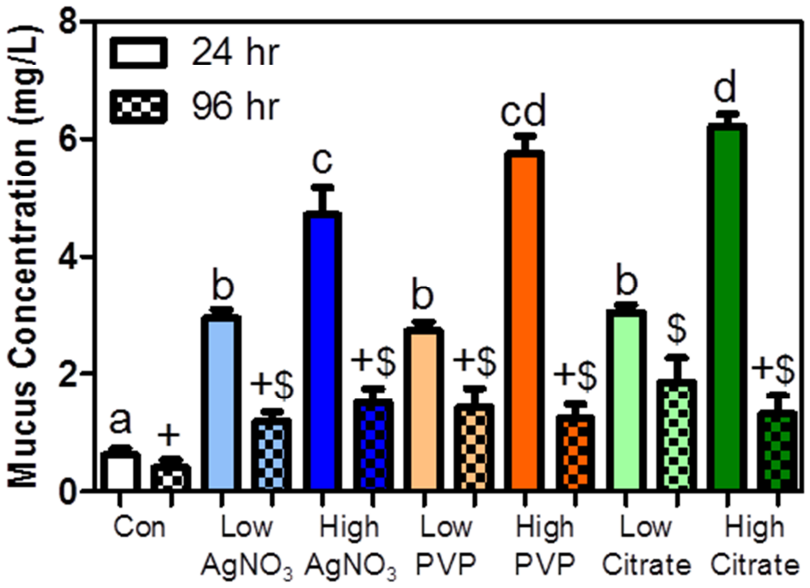


Figure 3.2. Mucus water concentration (mg/L) following 24 and 96 hr exposure. Water samples were collected at 24 and 96 hr to determine mucus concentration secreted into the exposure water using glucose as a surrogate. Statistical analysis was performed between treatments within time points. Bars with different letters (24 hr) or symbols (96 hr) are significantly different (p<0.05; ANOVA, Tukey post hoc; n=5 chambers/treatment).

5. Discussion

The presence of mucus in the unstirred layer of the fish gill is vital for protection of the gill structure and is critical for some gill functions (Shephard 1994). Mucus is able to reduce the toxicity of metals because of the presence of negatively charged sialic acid groups that can bind to the harmful cations of metals (Speare and Ferguson 2006). Furthermore, mucus secretion is stimulated quite quickly (within minutes to hours) upon metal exposure as shown previously in aluminum-treated rainbow trout (Handy et al. 1989). While gill mucus is able to effectively bind metals and reduce toxicity, it can also lead to the accumulation of metals at the main site of action of metal toxicity (Satchell 1984). The decreased ability of mucus production and, in turn, decreased mucus sloughing at the gill surface can lead to increased toxicity with prolonged exposure to silver.

The previous application of this glucose surrogate method for sloughed mucus quantitation utilized Atlantic silversides (*Menidia menidia*) wherein a mucus production rate of 567 µg mucus/hr/fish was determined (Parrish and Kroen 1988). The control FHMs in this study produced 44 µg mucus/hr/fish. The variation between studies can be explained by size differences between Atlantic silversides (~15 cm in length) and FHMs (averaged 5.3 cm). However, in our study the method was applied for the first time to understand mucus production after a toxicant exposure. Silver exposure increased mucus production and sloughing at 4 hrs to 390, 372, or 358 µg mucus/hr/fish exposed to 13 µg/L $AgNO_3$, 208 PVP-AgNPs, or 175 citrate-AgNPs, respectively.

The present study shows that control FHMs produced and replenished mucus at the same rate each day despite some predictable stress associated with handling and

daily water changes (Easy and Ross 2010). However, when fish were exposed to silver for 24 hrs they produced and sloughed a significantly greater amount (4.4 to 10-fold more) of mucus into the water. This sloughed mucus probably contained some Ag^{+} ions that were neutralized by sialic acid groups in mucus (Speare and Ferguson 2006) effectively reducing toxicity at first exposure. By 96 hrs of exposure, however, silver treated fish did not produce and slough a significantly greater amount of mucus than control fish at 96 hrs. This indicates that FHMs may have a reduced ability to neutralize the toxic effects of silver ions after perpetual exposure. The reduced ability to produce mucus at the cellular level is supported by our previous research that indicated that after 96 hrs of silver exposure in FHMs, mucus-secreting goblet cells are severely regressed in size (Chapter 2).

The reduced mucus water concentration could also be the result of the formation of a thickened mucus layer after silver exposure. In salmonids, a more viscous mucus coating was detected as fish were adapted from fresh to salt water with increased ionic strength (Roberts and Powell 2005). A thicker mucus layer is more difficult for the fish to slough and renew leading to an accumulation of xenobiotics and microorganisms. Also, thicker filaments decrease the ability of gill ionic regulation (Lichtenfels et al. 1996). Decreased mucus production also can reduce the ability of fish remove silver as water flow for oxygenation will be reduced across the gill (Speare and Ferguson 2006).

Mucus forms a protective physical barrier over the gills of fish preventing xenobiotic uptake and providing for an immune response. While initially $AgNO_3$ and AgNPs exposures triggered enhanced mucus production for acute protection, mucus renewal was not sustained in FHMs. Because mucus hypersecretion is a general stress

response and critical for protection from xenobiotic exposure, knowledge of the relationship between mucus production and sloughing after exposure is key to understanding pathologies and susceptibilities associated with each xenobiotic.

6. Acknowledgments

We would like to thank Frank Booc and Khalid Alharthy for assistance with water changes, feedings, and water sample collection. This research is supported by Army Corp of Engineers ERDC funding # W912HZ-09-C-0033.

CHAPTER 4. IDENTIFICATION OF SILVER NANOPARTICLES USING FLOW FIELD FLOW FRACTIONATION IN *PIMEPHALES PROMELAS* GASTROINTESTINAL TRACT AND GILL TISSUE

1. **Synopsis**

The environmental toxicity of silver nanoparticles (AgNPs) is of increasing concern due to their intensified production, use and subsequent environmental release. To further understand nanoparticle toxicity, more knowledge is needed about the particle fate upon uptake. AgNPs were identified in both the GI tract and gill of fathead minnows using Field-Flow-Fractionation interfaced to Inductively Coupled Plasma Mass Spectrometry (FFF-ICP-MS).

2. Methods, results and discussion

Silver nanoparticles (AgNPs) are among the most widely used nanoparticles and are included in products such as fabrics, washing machines, and medications. Due to the nature of these products their ultimate fate is waste water treatment plants and/or subsequent release into the environment (Sun et al. 2014). AgNPs are toxic to aquatic organisms due to the known toxicity of ionic silver (Shaw and Handy 2011). Yet, in some experiments, fish exposed to AgNPs have shown differing toxicities than those exposed to ionic silver indicating a unique particle effect (Griffitt et al. 2009). These

unique effects could be due to the differential bioaccumulation of AgNPs *in vivo* (Handy et al. 2008).

Understanding the relationship that nanoparticles have in toxicity requires an understanding of the particle form as it is taken up into the organism. Particle characterization in a tissue matrix offers a great challenge (Von der Kammer et al. 2012) because many analytical methods require dilution of the sample or an extraction of silver alters results due to transformation of the particles. For example, acid digestion of tissue is used to determine total silver concentrations, however, any particle-form information is destroyed and an understanding of the physiochemical properties is then impossible.

To meet these challenges, FFF-ICP-MS can be utilized to determine the presence, sizing, and possible heteroaggregation or homoaggregation of AgNPs in tissue of exposed organisms. FFF-ICP-MS was successfully employed to characterize polyvinylpyrrolidone (PVP) coated silver nanoparticles-AgNPs in *Lumbriculus variegates,* a freshwater oligochaete, which indicated an increase from 31 to 46 nm in hydrodynamic size of particles taken up by the worms (Poda et al. 2011). In the present work, FFF-ICP-MS was applied to the tissues of a freshwater fish.

Nanosilver release is of particular concern in freshwater ecosystems due the potential dissolution of particles into Ag^+ ions (Levard et al. 2012). While the fish gill is the major site of Ag^+ toxicity (Wood et al. 1999), unique biological targets of AgNPs toxicity could exist due to differing accumulation patterns (Shaw and Handy 2011). To understand the accumulation pattern of AgNPs in fish, fathead minnows (*Pimephales promelas,* FHM) were exposed to AgNPs and Ag tissue concentrations for gill and GI

tract were obtained. FFF-ICP-MS was also performed with tissues to characterize the particles detected in tissue.

Six to eight month old fathead minnows (length range 3.4 - 6.6 cm) were obtained from Aquatic BioSystems (Fort Collins, CO, USA) and cultured according to University of Mississippi IACUC approved conditions. Aqueous BioPure silver nanoparticle suspensions with the nominal size of 20 nm were supplied by Nanocomposix (San Diego, CA, USA). The fish were acclimated to glass chambers containing 1.5 L of moderately hard water (MHW) prepared by U.S. EPA guideline 821-R-02-013 for six days prior to the exposure. During acclimation, fish were fed and water was changed daily. The fish were then exposed to 6 µg/L $AgNO_3$ or PVP-AgNPs or citrate-AgNPs at nominally 200 µg/L for 96 hours (n=5 chambers/treatment; 3 fish/chamber; 1.5 L water/chamber). A single feeding occurred at 48 hrs, 30 min before water change. Water was changed and redosed daily at 9 AM. Water parameters were 286±4 µS, 189±3 ppm TDS, 26.6±0.7℃, 136±2 ppm sali nity, and pH 8.17±0.3. Water samples were taken at 20-30 min after dosing for concentration confirmation. The fish were euthanized with MS-222 and dissected. Body weight and length were recorded and gills and GI tract removed and frozen at -80℃ for FFF-ICP-MS analysis.

To verify differential tissue uptake of total silver, a replicate exposure was performed. Fish were exposed to measured water concentrations of 13.1±0.6, 208±40.7 and 175±5.2 µg/L of $AgNO_3$, PVP-AgNPs and citrate-AgNPs, respectively. Gill and GI tract were then removed and placed separately in 4 mL 50% nitric acid, 2.5 mL 30% hydrogen peroxide, and 0.5 mL water and refrigerated until ready for microwave digestion. An Ethos microwave digestion system (Milestone Inc. Shelton, CT, USA)

equipped with a 41-vessel rotor was employed for complete decomposition of the tissue. The digestion program consisted of a 30 min ramp to 120℃ followed by 15 min holding at that temperature. The resultant clear digests were diluted to 50 mL before ICP-MS analysis. Samples were then analyzed on the sector field-ICP-MS (Element XR, Thermo Fisher Scientific, USA) to test for silver accumulation (n=5 tanks; 2 fish per tank).

The results for the total Ag accumulation revealed that fathead minnows accumulated AgNPs in the GI tract more than in the gill. Ratios of concentrations (GI tract:gill) were 23:1, 17:1 and 0.44:1 for fish exposed to PVP-AgNPs, citrate-AgNPs, and $AgNO_3$, respectively. GI tract concentrations were 5.6±4.2 and 8.0±7.1 µg/g, and in the gill they were 0.24±0.05 and 0.48±0.19 µg/g, for PVP-AgNPs and citrate-AgNP, respectively.

In the replicate exposure of fish for the FFF analyses, the measured silver water concentrations were 13.1±0.6, 171±18.6 and 212±5.7 µg/L for $AgNO_3$, PVP-AgNP, and citrate-AgNP, respectively. Particles sizes in the exposure matrix of MHW were determined by FFF to be 26 nm for PVP-AgNPs and 27 nm for citrate-AgNPs. It was expected that the hydrodynamic based FFF method would measure slightly larger sizes compared to the nominal size of 20 nm which was measured by spectroscopic techniques (Poda et al. 2011).

GI tracts and gills extracted from fish for FFF-ICP-MS were placed in 600 µL of deionized water and the tissue was sonicated at 60% amplitude by a tissue demembraner (Fisher Science) for a total 65 sec with 5 sec pulses between 50 sec rest periods. Samples were then centrifuged at 6339 x *g* for 13 min and 250 µL was

analyzed for particle sizing. Particle sizing was performed using an F-1000 symmetrical flow field flow fractionation (FFF) system from Postnova Analytics (Salt Lake City, UT, USA), interfaced to a Perkin Elmer Elan DRC II ICP-MS using a MiraMist pneumatic nebulizer, with both ^{107}Ag and ^{197}Au monitored for metal nanoparticle detection (Bednar et al. 2013). The UV absorption data was collected using a Prominence UV/VIS detector from Postnova Analytics, primarily for detection of polystyrene bead size standards. UV absorbance data was not collected for the dilute nanosilver particles measured due to the limited absorbance of the silver nanoparticles at the low concentrations (µg/L) studied. The FFF system was equipped with a 10 kDa regenerated cellulose membrane. The mobile phase consisted of a 0.01% sodium azide and 0.01% FL-70 surfactant dissolved in deionized water with a resistivity of 18.3 MΩ. Separation of the particles under investigation was achieved using a channel flow of 1.0 mL/min and a cross flow of 0.5 mL/min. The channel flow conditions allow direct connection of the FFF effluent to the ICPMS nebulizer without a flow splitter.

AgNPs were identified in the GI tract of fish exposed to both PVP-AgNPs and citrate-AgNPs (Figure. 4.1) using FFF-ICP-MS. For PVP-AgNP exposures (Figure 4.1A) the particles agglomerated more with some particles found near the original size of 25 nm but others at 40-70 nm. Fish exposed to citrate-AgNPs displayed a single peak in the fractogram (Figure 4.1B) with AgNPs ranging from 40-55 nm in size. As expected, GI tracts from fish in the $AgNO_3$ group displayed no peaks in the fractogram after the void peak (Figure 4.1C). The agglomeration pattern in both PVP-AgNPs and citrate-AgNPs could be due to the relative stability of each particle coating in the water column prior to uptake and after ingestion. Sterically stabilized PVP-AgNPs are generally more

stable in complex environmental media than the charge-stabilized citrate-AgNPs (Yu et al. 2013). PVP-AgNPs could be maintaining their particle form but with the formation of a protein corona or heteroaggregating with other biological media resulting in a greater range of (hydrodynamic) particle sizes (Lin et al. 2012). Citrate-AgNPs are more likely homoaggregate first forming larger silver particles that result in less interaction with biological media and reduced diversification in particle size.

AgNPs were also identified in the gill tissue of fish exposed to both PVP-AgNPs and citrate-AgNPs (Figure 4.2) using FFF-ICP-MS. After PVP-AgNPs exposure, the particles did not agglomerate in gill displaying only a single peak sizing at 27 nm (Figure 4.2A). Fish exposed to citrate-AgNPs (Figure 4.2B) also only had a single peak with AgNPs at 30 nm in size. AgNPs in gill tissue were less aggregated than those found in GI tissue, probably due to fewer interactions with biological substances and overall lower AgNP concentrations. Furthermore, AgNPs found in gill tissue were likely indicative of more recent (e.g. within the last 24 hr of the 96 hr exposure) as compared to particles in the GI which may have accumulated throughout the exposure. The mucus produced by the gill can provide a mechanism by which AgNPs are sloughed off thus limiting gill bioaccumulation. This observation was supported by relatively lower AgNP concentrations in gill relative to GI tract. Conversely, AgNPs in GI tissue can be absorbed over time due to stress induced drinking (Shaw and Handy 2011).

The synthesis of AgNPs often employs the use of gold NP ‘seeds’ as indicated by the gold trace apparent in the FFF-ICP-MS analysis in Figures 4.1 and 4.2. Because the AuNP is resistant to dissolution, unlike AgNPs, it can be used as a particle tracking mechanism. The gold signal indicates that the NPs remain intact and the silver trace is

not merely ionic silver associated with large organic moieties or debris. The gold ‘tracer’ can be clearly seen in Figure 4.1A and B, where substantial amounts of AgNPs are observed. However, where the NP signature is 1-2 orders of magnitude less in Figures 4.2A and B, the gold signature is at or near the detection limit in gill tissue exposures.

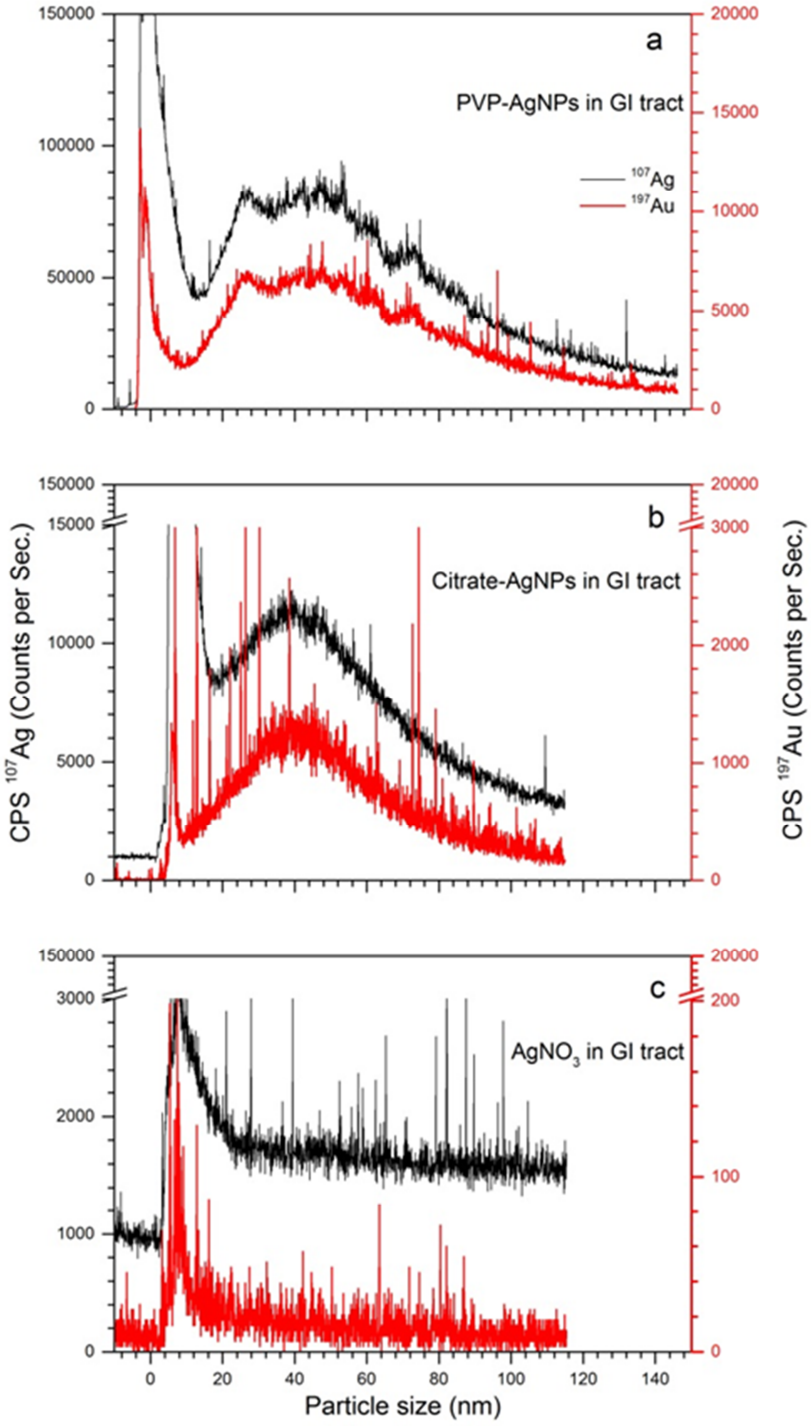


Figure 4.1. FFF-ICP-MS fractograms of the GI Tract of FHMs after exposure to (a) PVP-AgNPs, (b) citrate-AgNPs, or (c) $AgNO_3$

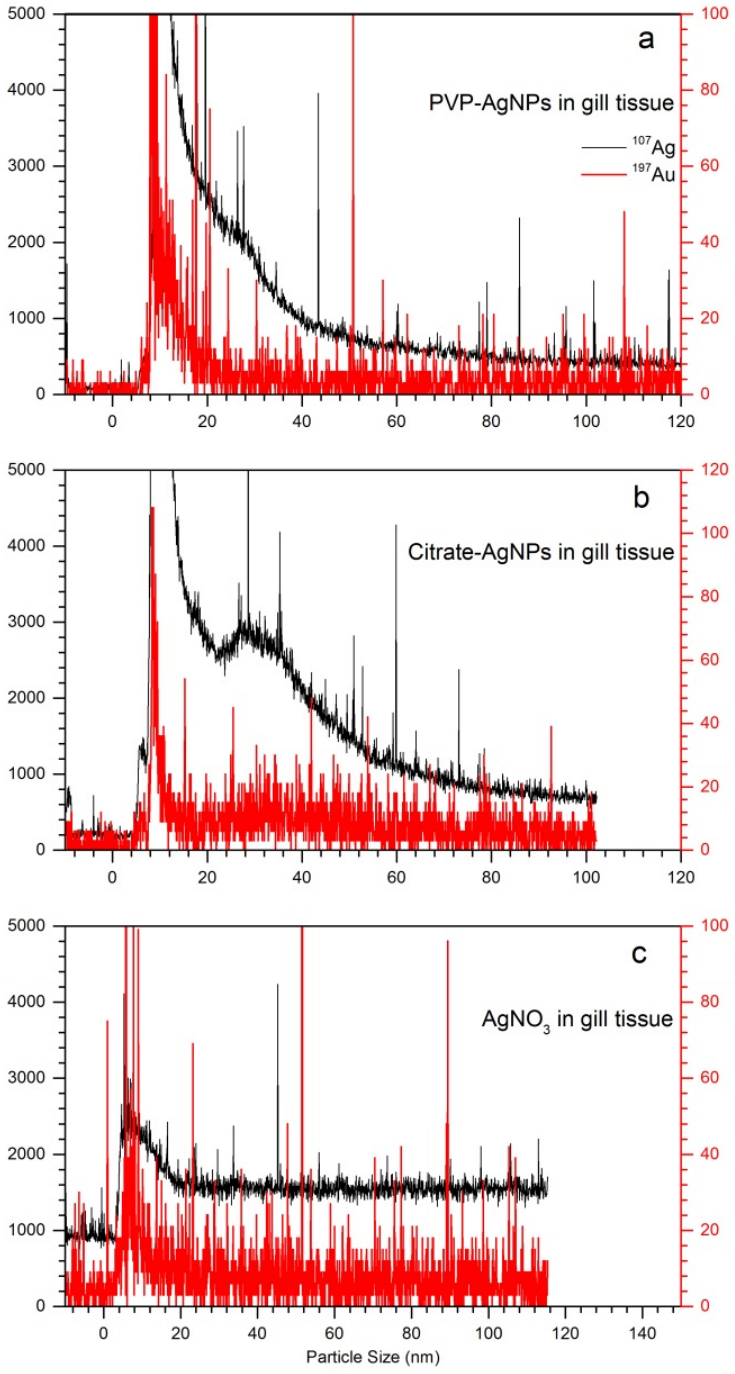


Figure 4.2. FFF-ICP-MS fractograms of the gill of FHMs after exposure to (a) PVP-AgNPs, (b) citrate-AgNPs, or (c) $AgNO_3$

FFF-ICP-MS can be a useful tool in understanding particle fate after uptake. Some limitations exist, however, because the nanoparticles must be in high enough concentrations. A detection limit of 10 µg/L has been reported for FFF-ICP-MS analysis of water (Poda et al. 2011; Bednar et al. 2013). FFF analysis of *Lumbriculus* that averaged 4.4±1.9 µg/g total Ag had no detectable particle signal (Coleman et al. 2013). The total Ag concentration range in *Lumbriculus* was comparable to GI tract concentrations reported here, however the fish samples were extracted in only 600 µl compared to the 10 mL used for *Lumbriculus* prior to FFF analysis. There is an analytical trade off in that minimal dilution of samples results in a viscous sample for injection into FFF-ICP-MS, whereupon significant fouling of the FFF membrane with biomolecules can occur and interfere with particle elution.

3. Conclusions

This research was able to confirm that AgNPs can be characterized in the GI tract and gill tissue of exposed fish using FFF-ICP-MS. This method could be a useful in understanding the bioaccumulation and speciation of nanoparticles following *in vivo* or *in vitro* exposures. Gold seeded silver nanoparticles can be an insightful tool because the gold core is important in confirming the presence of a particle form and provides data on the fate of the nanoparticle in such complex systems.

CHAPTER 5. TISSUE ACCUMULATION AND ALTERED GENETIC EXPRESSION IN FATHEAD MINNOWS AFTER SILVER NITRATE OR AgNP EXPOSURE

1. Synopsis

Concerns arise from increased silver nanoparticles (AgNPs) use not only because of the known toxicity attributed to the release of Ag^+ ions but also the potential of unique particle effects and biological targets. To identify unique biological targets and particle specific gene expression alterations, adult fathead minnows were exposed to 20 nm citrate- or polyvinylpyrrolidone (PVP)-coated AgNPs at nominal concentrations of 20 or 200 µg/L and $AgNO_3$ at 2 or 6 µg/L. After 96 hr exposure, gill, skin, liver, GI tract, and brain were acid digested and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for silver accumulation. Despite greater total silver water concentrations in AgNPs, $AgNO_3$ accumulated to greater concentrations in gill but had similar concentrations to the AgNPs in the skin and liver. AgNPs accumulated to the greatest concentrations in the GI tract indicating a potential unique biological target of AgNP exposure. No accumulation in brain tissue was above the detection limits for any treatment. Because the known site of toxicity of the Ag^+ ion is the fish gill, RNA microarrays were performed on exposed fish. Microarray results suggested possible unique particle effects because there were 1038 differentially expressed genes (DEGs) unique to AgNP in gill tissue. Pathway analysis also revealed possible unique particle effects because 82 unique toxicity pathways were enriched after AgNP exposure.

Future work should investigate the GI gene expression because of the unique particle accumulation found in that tissue.

2. Introduction

Most of the toxicity of AgNPs can be attributed to the Ag^+ ion (Newton et al. 2013; Kennedy et al. 2010). However, unique particle effects are possible due to direct particle interaction with biological targets (Griffitt et al. 2009; Shaw and Handy 2011). Ag^+ ions are known to accumulate in fish tissue with the gill being the main site of uptake (Wood et al. 2004; Long and Wang 2005). AgNPs have been visualized by hyperspectral imaging in gill, brain, and GI tract of Japanese medaka (*Oryzias latipes*) embryos after 24 h of exposure (Kwok et al. 2012). Carp (*Cyprinus carpio*) accumulated AgNPs in liver, GI tract, and gills after a 21 d exposure (Gaiser et al. 2012). Juvenile rainbow trout (*Oncorhynchus mykiss*) also accumulated AgNPs in gill and liver after 10 d of exposure (Scown et al. 2010).

Though studies have investigated differential gene expression between fish exposed to AgNPs and ionic silver using zebrafish gills (Griffitt et al. 2009) and brain and liver tissue from fathead minnows (Garcia-Reyero et al. 2014). The differences between genes differentially expressed by AgNPs and $AgNO_3$ indicated AgNPs have both toxicities associated with direct delivery of Ag^+ ions to different biological targets and particle effects. For example, in the fathead minnow study, pathway analysis revealed 77% of toxicity pathways were common between AgNP and $AgNO_3$ exposure indicating toxicity was derived from the Ag^+ ion in both treatments. Whereas the brain was identified as a possible unique target of AgNP exposure because there were only 42% of toxicity pathways common to $AgNO_3$ (Garcia-Reyero et al. 2014).

In this study, FHMs were exposed to three water concentrations of 20 nm citrate-AgNPs (10.1, 84, 175 µg/L), PVP-AgNPs (11.1, 75.4, 208 µg/L) or $AgNO_3$ (0.82, 5.7, 13.2 µg/L) to determine formulation-specific biological targets by understanding the accumulation pattern in gill, skin, liver, GI tract, and brain. Also, microarrays were performed to investigate particle-specific gene expression alterations in the fish gill, the main site of Ag^+ toxicity.

3. Materials and methods

3.1 Fish source and handling

Fathead minnows (*Pimephales promelas*) at six to eight months old (length 3.2 – 6.7 cm) were obtained from Aquatic BioSystems (Fort Collins, CO, USA) and cultured in 30 L tanks until needed for exposure according to University of Mississippi IACUC approved conditions. Prior to exposure, fish were allowed to acclimate in glass exposure chambers containing 1.5 liters of moderately hard water (MHW) prepared by U.S. EPA guideline 821-R-02-013 with daily water changes and fed Tetramin flakes (Blacksburg, VA, USA).

3.2 Silver

Biopure PVP-AgNPs and citrate-AgNPs were obtained from Nano Composix (San Diego, CA, USA) at a concentration of 1 mg/mL and a nominal size of 20 nm. Concentrated stock suspensions were prepared by sonicating the sample mixture in a water bath for 5 minutes and inverting to mix. Concentrated stocks were diluted with nanopure water to obtain a working stock with a nominal concentration of 40 µg/mL.

Particles were previously characterized (Chapter 2, Figure 2.1) resulting in primary particle sizes of 21±4 nm in citrate-AgNPs and 22±2 nm in PVP-AgNPs. Hydrodynamic sizing was also previously determined to be 29 nm in AgNPs as determined by FFF-ICP-MS. Commercially available silver nitrate was obtained (CAS 7761−88−8, Sigma Aldrich, St. Louis, MO) and diluted to a working stock in nanopure water with a nominal concentration of 10 µg/mL.

3.3 Exposure

Fathead minnows were exposed to control, three concentrations of PVP-AgNPs (11.1, 75.4, 208 µg/L), citrate-AgNPs (10.1, 84, 175 µg/L) or silver nitrate ($AgNO_3$; 0.82, 5.7, 13.2 µg/L) for 96 hours (n=5 chambers/treatment; 3 fish/chamber; 1.5 L water/chamber) in two separate exposures. The fish were fed once at 48 hr, 30 min before water change. Water was changed and redosed daily at 9 AM. The fish were euthanized with MS-222 and dissected. Body weight and length were recorded and gills, liver, GI tract, brain, and skin removed from the side of the fish between the pectoral fin and anal fin were removed. Gills were either placed in RNAlater (Qiagen, Valencia, CA, USA) and stored at -80℃ for microarray analysis or placed separately in 4 mL 50% nitric acid, 2.5 mL 30% hydrogen peroxide, and 0.5 mL water and refrigerated until ready for microwave digestion for total silver analysis. Similarly, skin, liver, GI tract, and brain were preserved in nitric acid as described above for total silver analysis. Water samples were collected at 10-30 min following dosing to confirm Ag concentration (n=5) and mixed with 5 mL 10% nitric acid and refrigerated until analysis.

3.4 ICP-MS

An Ethos microwave digestion system (Milestone Inc. Shelton, CT, USA) equipped with a 41-vessel rotor was employed for complete decomposition of the tissue. The digestion program consisted of a 30 minute ramp to 120℃ followed by 15 minutes holding at that temperature. The resultant clear digests were diluted to 50 mL before ICP-MS and analyzed for Ag using a sector field-ICP-MS (Element XR, Thermo Fisher Scientific, USA). Sample and standard solutions were introduced via a concentric glass nebulizer coupled to a glass spray chamber. Rhodium was added online as internal standard. External calibration was performed using a Ag standard ranging from 0.1 ppb to 20 ppb (SPEX CertiPrep, USA).

3.5 RNA microarray

Total RNA was isolated from samples using RNeasy kits (Qiagen, Valencia, CA, USA) and DNAase treated. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The RNA quantity was determined using a Nanodrop®ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Total RNA was stored at -80^{o}C until analyzed using oligonucleotide microarrays. Custom fathead minnow 60,000-probe microarrays were purchased from Agilent (Palo Alto, CA). Array hybridizations were performed using a single color design. The cDNA synthesis, cRNA labeling, amplification and hybridizations were performed following the manufacturer's kits and protocols (One Color Microarray-based Gene Expression Analysis Quick Amp Labeling version 5.7; Agilent, Palo Alto, CA). After hybridizing for 17 h, microarrays were washed and then scanned with a Surescan High-Resolution DNA Microarray scanner G2505 C (Agilent, CA, USA). Data was extracted from

microarray images using Feature Extraction software (Agilent, CA, USA). Raw microarray data was imported into Genespring version GX11 (Agilent, Santa Clara, CA, USA). Raw data was normalized using quantile normalization, followed by median scaling across all samples. To identify genes that are most variable between the control and the exposure, the one-way Analysis of Variance (ANOVA) test was performed followed by pair-wise Tukey's HSD (Honestly Significant Difference) test ($p<0.05$). Functional analysis of DEGs was performed using Ingenuity Pathway Analysis (IPA, Redwood City, CA, USA).

4. Results

4.1 Silver water concentrations

Total silver water concentrations in exposures for tissue Ag accumulation and microarray analysis (exposure 1) were 5.7, 75.4, and 84 µg/L for $AgNO_3$, PVP-AgNPs, and citrate-AgNPs, respectively. A subsequent exposure (exposure 2) was performed adding a dose response for tissue accumulation including two doses of $AgNO_3$ (0.82 or 13.2 µg/L), PVP-AgNPs (11.1 or 208 µg/L) and citrate-AgNPs (10.1 or 175 µg/L).

4.2 Silver tissue accumulation

No fish accumulated silver in the brain above the level of detection of our instrumentation. In all other tissues, silver concentrations showed high variability resulting in difficulty assigning statistical significance between silver-treated fish (n=5). In most cases, the variability was lowest in control and the lowest exposure concentration of each treatment. In contrast, tissues of fish exposed to 175 µg/L citrate-AgNPs, a range of 84-36,000, 110-1000, 300-2400, and 31-1000 ng/g were present in

GI tract, gill, liver, and skin, respectively (Figures 5.1-5.4). While accumulation trends were not significantly different, ratios were used to determine patterns between treatments and unique biological targets.

For gill tissue accumulation, data only exist for the highest and lowest concentration in each treatment because gills from the middle dose were used for the microarray studies. Silver accumulation in gill was significantly greater than control in 13.2 µg/L $AgNO_3$, 208 µg/L PVP-AgNPs, and 175 µg/L citrate-AgNPs (Figure 5.1). The lower silver concentrations in each treatment were not significantly different from control or the higher silver concentrations in each treatment. While not significantly different from other silver-treated fish, 13.2 µg/L $AgNO_3$ had the greatest Ag concentration with an average of 841 ng/g.

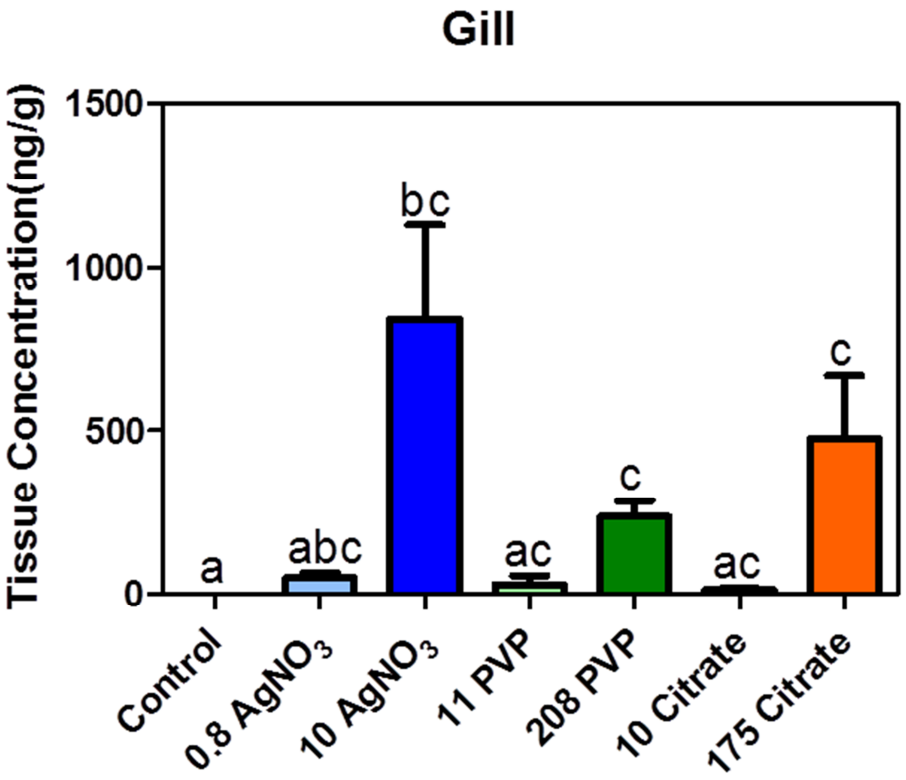


Figure 5.1. Silver concentrations in gill tissue after exposure to AgNPs or $AgNO_3$. Different letters indicate significant difference (log transformed; Kruskal-Wallis, n=5).

Silver concentrations in skin tissue were significantly higher than control in 6 µg/L $AgNO_3$, 75 µg/L PVP-AgNPs, and 84 µg/L citrate-AgNPs (Figure 5.2). However, 175 µg/L citrate-AgNPs had the greatest average tissue concentration at 232 ng/g but was not significantly different than controls due to variability within the treatment. In the liver, 0.8 and 13 µg/L $AgNO_3$, 11 µg/L PVP-AgNP, and 10 µg/L citrate-AgNPs did not accumulate silver significantly greater than controls, while accumulation in all other treatments was significantly higher (Figure 5.3). Unexpectantly, the maximum average

concentration detected in the liver was ~1500 ng/g and this was found in the middle concentrations of $AgNO_3$ (6 µg/L) and citrate-AgNPs (84 µg/L).

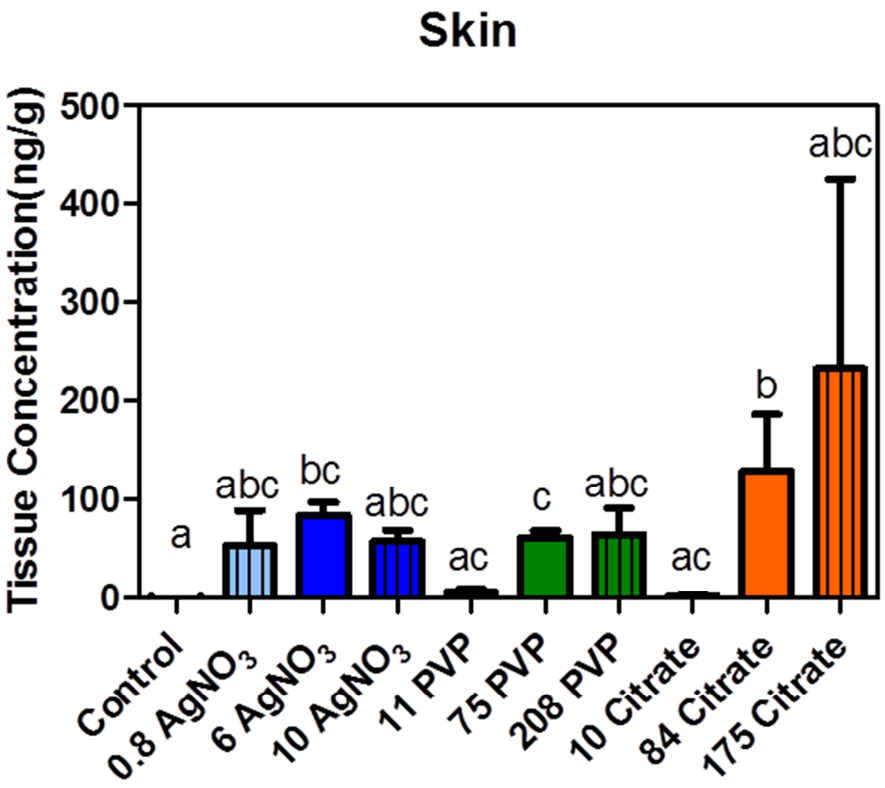


Figure 5.2. Silver concentrations in skin after exposure to AgNPs or $AgNO_3$. Different letters indicate significant difference (log transformed; Kruskal-Wallis, n=5-10).

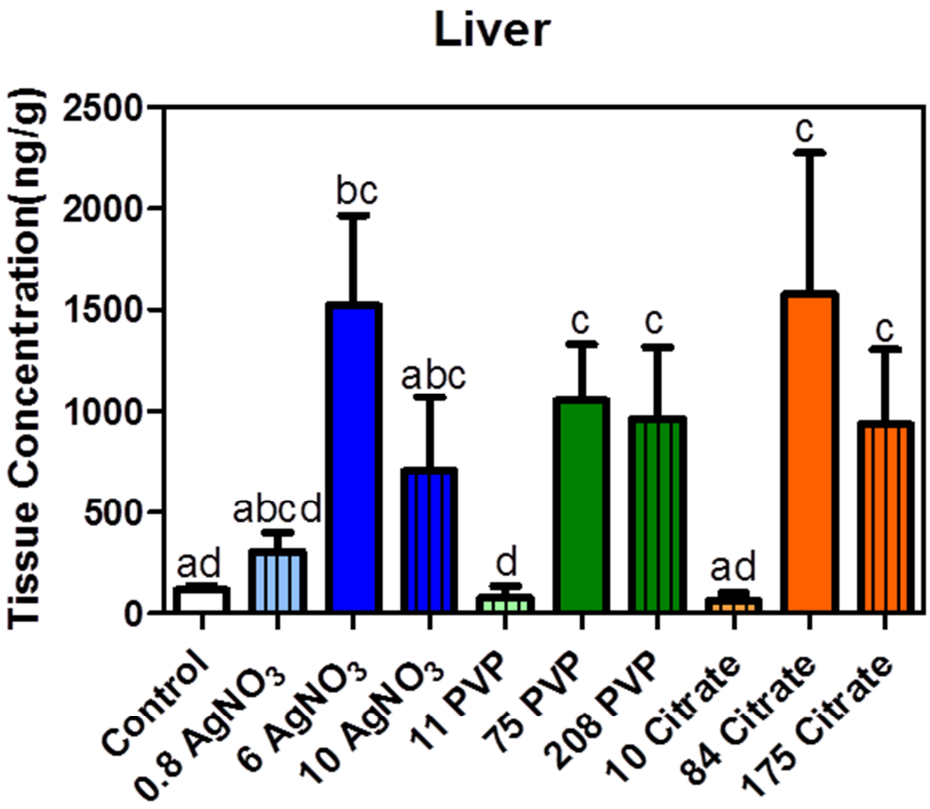


Figure 5.3. Silver concentrations in liver tissue after exposure to AgNPs or $AgNO_3$. Different letters indicate significant difference (log transformed; Kruskal-Wallis, n=5-10).

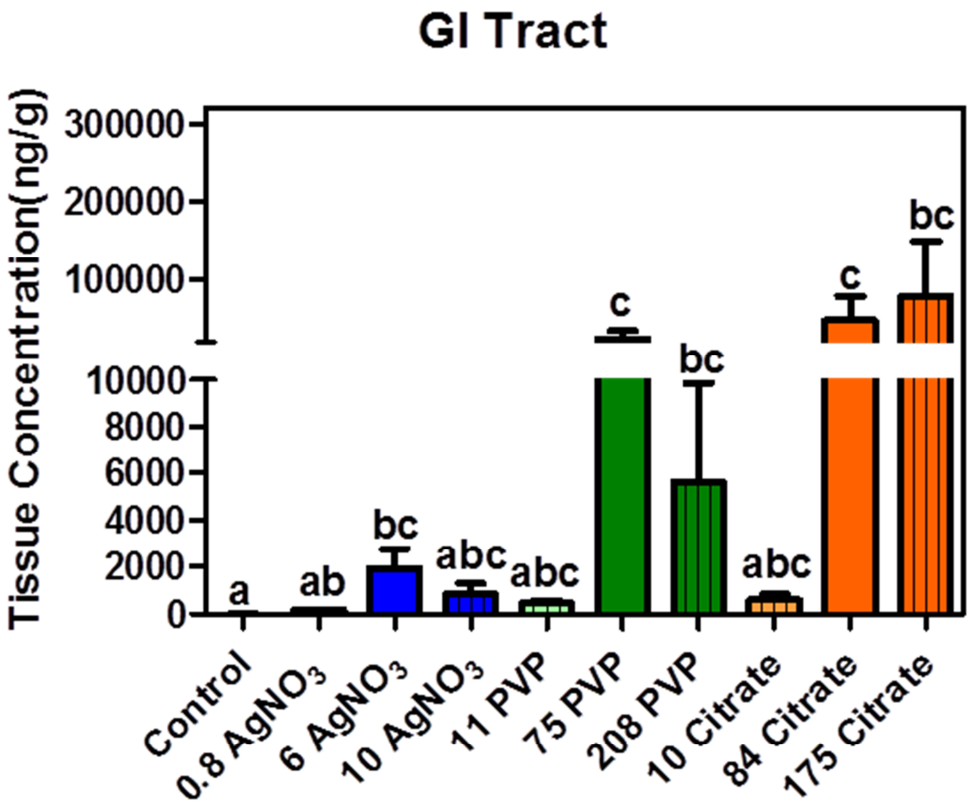
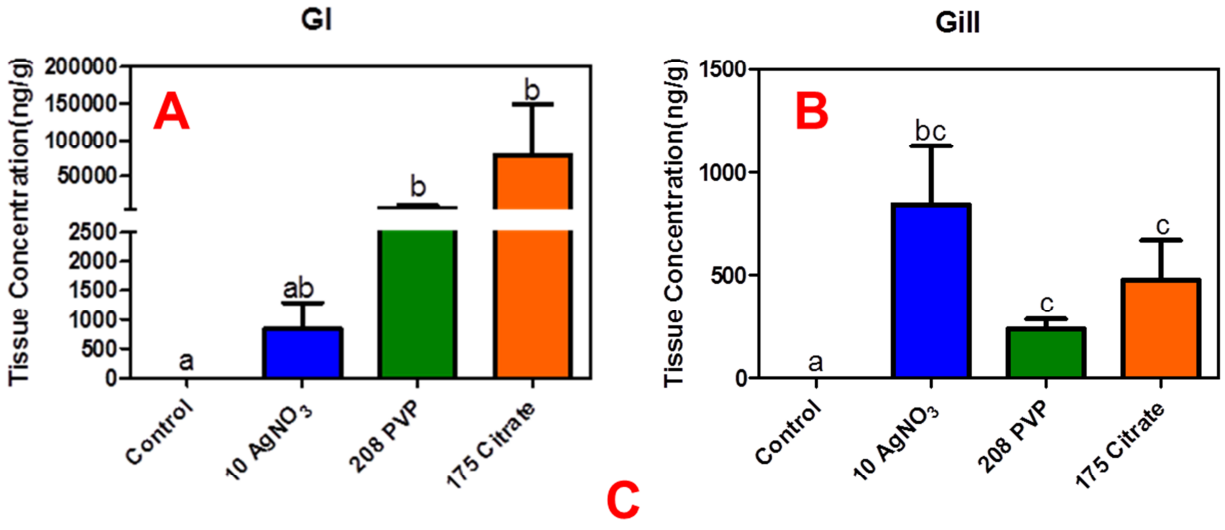


Figure 5.4. Silver concentrations in GI Tract tissue after exposure to AgNPs or $AgNO_3$. Different letters indicate significant difference (log transformed; Kruskal-Wallis, n=5-10).

GI tract accumulated silver significantly more than controls in the two highest concentrations of both AgNPs and the 6 µg/L $AgNO_3$ (Figure 5.4). While not significantly different, AgNPs-exposed fish accumulated silver in the GI tract at greater than 10-fold higher than $AgNO_3$ in the highest concentrations (Figure 5.5A). When the highest concentrations of exposure were considered for both the GI tract and gill tissues, a pattern was present where despite higher exposure concentrations in AgNP fish, the $AgNO_3$ exposed fish accumulated silver preferentially in the gill. Conversely, AgNP-exposed fish accumulated silver to a much greater extent in the GI tract (Figure 5.5A, B). To understand the relationship of GI tract to gill accumulation, ratios were calculated

for all treatments (Figure 5.5C) and indicated an AgNP accumulation pattern that was ~20-fold higher in GI tract compared to gill (Figure 5.5C).



C

Treatment	Ratio (GI Tract:Gill)
PVP-AgNPs	23:1
Citrate-AgNPs	17:1
$AgNO_3$	0.44:1

Figure 5.5. Pattern and ratio of accumulation of GI tract and gill tissue. Accumulation of silver in GI tract (A) or gill (B) in FHM exposed to the high treatment concentrations revealing differential patterns of accumulation between AgNP and $AgNO_3$ -exposed fish and confirmed by the calculated ratios between tissue uptake (C).

4.3 Gill microarray

Gill tissues from FHMs exposed for 96 hr to 84, 75, or 5.7 µg/L of citrate-AgNPs, PVP-AgNPs, or $AgNO_3$, respectively were analyzed by RNA microarrays and Ingenuity Pathway Analysis (IPA). Hierarchical analysis of FHM gill samples showed distinct treatment differences in gene expression with individuals from each treatment clustering

together (Figure 5.6). Among treatments, citrate-AgNPs had the most differential gene expression with 70% (615 of 823 total DEGs) of altered compared to the $AgNO_3$ and PVP-AgNPs (Figure 5.7A).

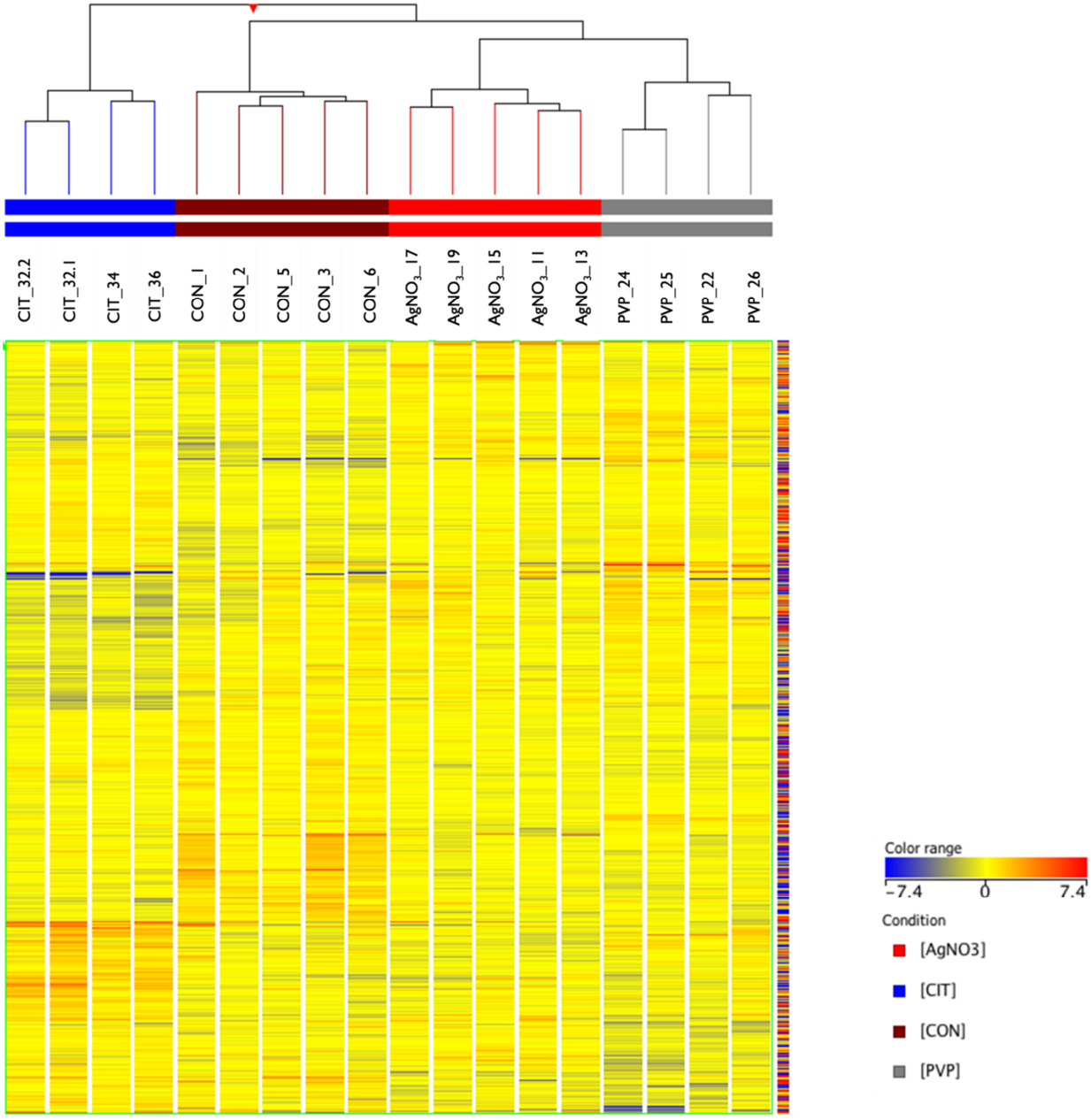


Figure 5.6. Hierarchical clustering of gene expression in the gills of individual fathead minnow males exposed to control, $AgNO_3$, PVP-AgNPs or citrate-AgNPs.

PVP-AgNPs and $AgNO_3$ had unique altered differential expression gene expression of 57% (423 of 741 total DEGs) and 40% (185 of 463 total DEGs), respectively. Of the genes differentially expressed, 5% (110) were common among all silver treatments and 10% (208) were common among AgNPs exposed fish (Figure 5.7A). Using IPA to examine biological pathways, citrate-AgNPs had the highest number of unique enriched pathways with 45% (62; Figure 5.7B). Citrate-AgNPs and $AgNO_3$ had the most similar enriched pathway profile with 23% (70) common pathways while AgNPs only shared 11% (31) common pathways.

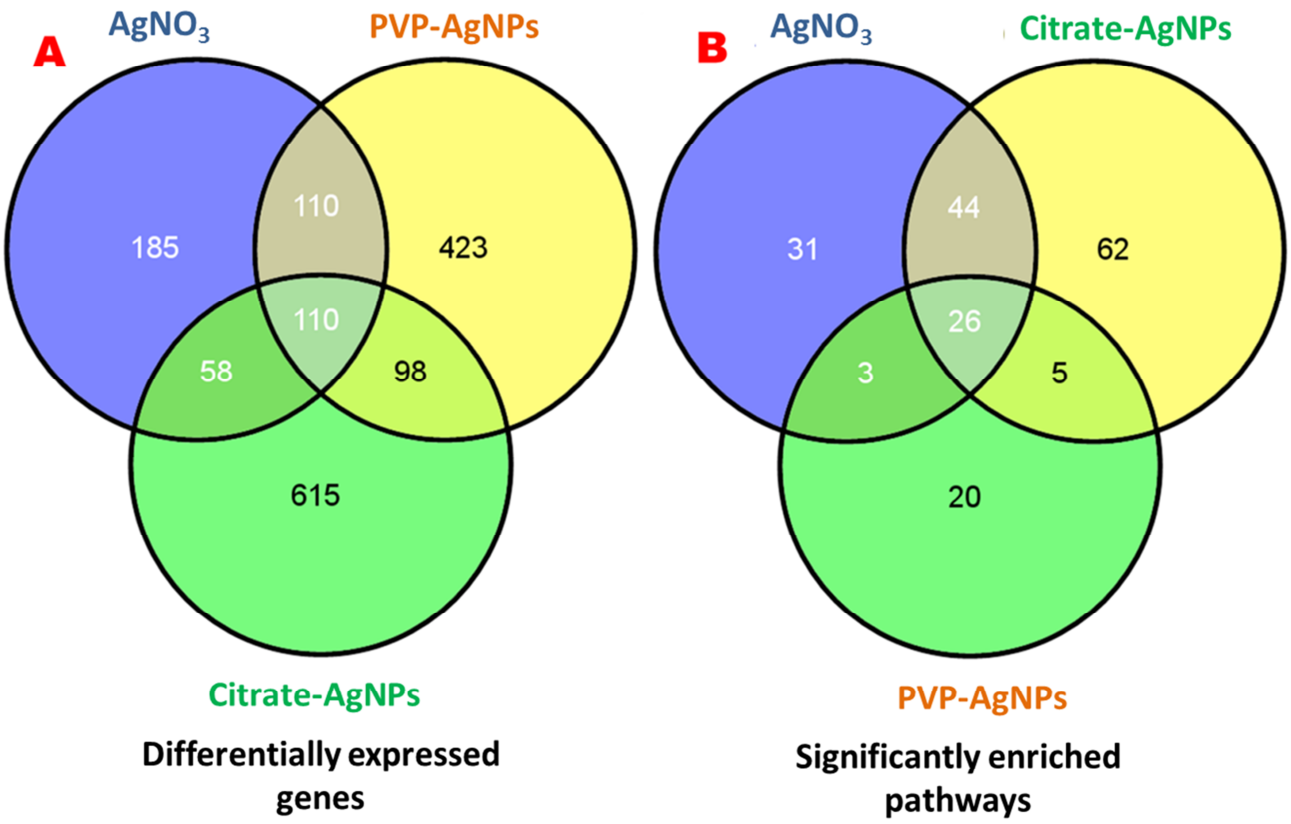


Figure 5.7. Venn diagram showing number of common and distinct differentially expressed genes (DEGs;A) or common and distinct significantly enriched pathways (B) in the gill of fathead minnow males exposed to $AgNO_3$, PVP-AgNp or citrate-AgNP.

Table 5.1. The most upregulated and downregulated genes in gill of fish exposed to $AgNO_3$ compared to controls.

	$AgNO_3$		
Gene Symbol	Description	P value	Fold change
MR1	Major Histocompatibility Complex Class I UFA Gene / Major Histocompatibility Complex Class I UXA2 Gene /	0.003595	309
MHC1UXA2	major histocompatibility complex class I UXA2	0.004127	233
KRT1-19D	novel protein similar to vertebrate keratin family	8.88E-04	16.3
	Zgc:113691 /	0.006539	10.9
		0.003274	8.99
C9ORF98	Zgc:112030 /	0.028884	7.55
SI:DKEY-96L17.6	PREDICTED: si:dkey-96l17.6	0.029458	7.08
GLS	Hypothetical LOC564746 /	0.010115	6.47
PDZRN4	PDZ domain containing ring finger 4	0.017161	6.25
C3ORF25	Hypothetical Protein LOC798261 /	0.004222	5.88
PSMB9	proteasome subunit beta type-9	0.043114	-237
PSMB9	Proteasome Subunit, Beta Type, 9a /	0.047007	-169
IRG1L	immunoresponsive gene 1, like	0.008729	-16.6
IRG1L	immunoresponsive gene 1, like	0.008418	-14.6
NOS2	novel protein similar to vertebrate nitric oxide synthase 2A	0.010057	-13.5
IRG1L	immunoresponsive gene 1, like	0.021354	-10.6
WU:FJ08F03	wu:fj08f03	0.020266	-9.73
IRG1	Zgc:154020 /	0.034722	-9.7
	Hypothetical LOC556026 / Hypothetical Protein LOC100002183 /	0.005584	-8.69
CLCA4	Similar To Clca1 Protein /	0.001038	-8.1

Table 5.2. The most upregulated and downregulated genes in gill of fish exposed to PVP-AgNPs compared to control.

PVP-AgNPs			
Gene Symbol	Description	P value	Fold change
MR1	Major Histocompatibility Complex Class I UFA Gene / Major Histocompatibility Complex Class I UXA2 Gene /	0.003595	439
MHC1UXA2	major histocompatibility complex class I UXA2	0.004127	358
NARS	PREDICTED: asparaginyl-tRNA synthetase, cytoplasmic	0.007961	299
TXNDC14	Thioredoxin Domain Containing 14 /	0.009607	141
TXD14	Thioredoxin domain-containing protein 14 precursor	0.008343	119
LOC793474	PREDICTED: hypothetical protein LOC793474	0.005681	37.9
IG	immunoglobulin light chain	0.02244	20
GAL	Similar To Preprogalanin 1A /	0.012217	15.1
PLCB2	phospholipase C-beta 2	0.033457	12.9
LOC100006361	PREDICTED: galanin-like	0.012747	11.2
GSR	Glutathione reductase, mitochondrial precursor	0.010964	-68.9
GSR	Glutathione Reductase /	0.008847	-62.6
LOC100534786	uncharacterized LOC100534786	4.95E-05	-48.5
NDUFV1	NADH Dehydrogenase Flavoprotein 1 /	0.006742	-38.3
LOC798684	hypothetical protein LOC798684	0.001839	-29.5
	Hypothetical LOC559555 /	0.003929	-25.2
RAB1	mKIAA3012 protein	0.009803	-19.4
CASP2	caspase-2	0.014139	-18.9
	MHC class I antigen	0.040377	-15.8
CASP2	caspase-2	0.011126	-13.1

Table 5.3. The most upregulated and downregulated genes in gill of fish exposed to citrate-AgNPs compared to control.

Citrate-AgNPs			
Gene Symbol	Description	P value	Fold change
TXNDC14	Thioredoxin Domain Containing 14 /	0.009607	133
TXD14	Thioredoxin domain-containing protein 14 precursor	0.008343	110
0PRB3	Hypothetical LOC557373 /	0.003418	52.4
SI:CH211-182E10.4	hypothetical protein LOC553308	0.00175	45.4
LOC100148318	PREDICTED: hypothetical protein LOC100148318	0.012409	28
CEL	Cel.2 protein	0.006976	24.8
ICA	islet cell autoantigen 1-like protein	0.005409	17.9
	unnamed protein product	0.003285	12
CEL	Carboxyl Ester Lipase, Tandem Duplicate 2 /	9.55E-04	11.1
LOC794998	novel protein	0.005855	10.2
ZGC:92161	hypothetical protein LOC447817	5.45E-04	-141
PLBD1	phospholipase B domain containing 1	0.005212	-71.1
	Zgc:92161 / Hypothetical Protein LOC791539 / Zgc:163083 /	0.001002	-59.1
LOC100334396	serine/threonine-protein kinase ppk4-like	0.041648	-57.2
PIGU	Phosphatidylinositol Glycan Anchor Biosynthesis, Class U /	0.048819	-50.8
LOC797583	PREDICTED: uncharacterized protein K02A2.6-like	0.032902	-28.8
ISOC2	isochorismatase domain-containing protein 2	9.63E-05	-23.6
ISOC2	Zgc:109735 /	9.43E-04	-16
M6PR	cation-dependent mannose-6-phosphate receptor	0.00768	-15.6
CASP2	caspase-2	0.014139	-15.3

Analysis of gene expression showed that the MR1 and MHC1UXA2 genes, which encode major histocompatibility complex (MHC) on the cell surface, were the most upregulated genes in both fish exposed to $AgNO_3$ (310 and 233-fold; Table 5.1) and PVP-AgNPs (440 and 358-fold; Table5.2). Fish exposed to citrate-AgNPs had induced expression of TXNDC14 and TXD14 (133 and 110-fold; Table 5.3) in gill. These genes participate in various redox reactions. Moreover, fish gill exposed to either PVP-AgNPs or citrate-AgNPs showed decreased expression of Casp2 (-19 and -15-fold, respectively), a mediator of apoptosis and tumor suppressor. The most highly downregulated gene from $AgNO_3$ exposure was PSMB9 (-237 fold). PSMB9 is responsible for proteasome function in the proteasomal degradation pathway. This pathway is essential for many cellular processes including cell cycle, regulation of gene expression, and responses to oxidative stress.

Pathways analysis revealed that the most enriched pathway was cell cycle control of chromosomal replication regardless of silver treatment. Moreover, among the ten most enriched pathways in all treatments, eight were in common between PVP-AgNPs and $AgNO_3$ and six were common among citrate-AgNPs and $AgNO_3$ (Table 5.4). The functions of the common pathways indicated that all exposures ($AgNO_3$, PVP-AgNPs, or citrate-AgNPs) impacted cell cycle, cell proliferation, protein ubiquitination and apoptosis signaling pathway genes. Citrate-AgNPs affected the highest number of toxicity pathways. Commonly affected pathways following PVP-AgNPs and citrate-AgNPs exposures were DNA repair and melatonin pathways.

Table 5.4. Common enriched pathways in the gills of fish exposed to $AgNO_3$, PVP-AgNPs, and citrate-AgNPs.

Ingenuity Canonical Pathways	$AgNO_3$		PVP-AgNPs		Citrate-AgNPs	
	Rank	P Value	Rank	P Value	Rank	P Value
Cell Cycle Control of Chromosomal Replication	1	1.74E-07	1	1.58E-11	1	3.47E-07
ATM Signaling	2	3.24E-07	8	0.000501	4	2.69E-06
Aryl Hydrocarbon Receptor Signaling	3	4.37E-07	10	0.000692	7	5.37E-06
IL-17A Signaling in Gastric Cells	4	1.78E-06	-	-	9	3.24E-05
Role of CHK Proteins in Cell Cycle Checkpoint Control	5	1.86E-06	4	5.01E-06	25	0.00049
Molecular Mechanisms of Cancer	7	2.34E-06	11	0.001698	2	1.38E-06
Role of BRCA1 in DNA Damage Response	8	3.72E-06	2	1.32E-07	28	0.000832
Mismatch Repair in Eukaryotes	9	6.17E-06	3	2.4E-06	6	3.89E-06
p53 Signaling	10	2.04E-05	9	0.000501	12	4.17E-05
Protein Ubiquitination Pathway	-	-	5	1.32E-05		
Induction of Apoptosis by HIV1	-	-	7	0.000447	3	2.29E-06
Circadian Rhythm Signaling	-	-	-	-	5	2.82E-06
4-1BB Signaling in T Lymphocytes	-	-	-	-	8	1.07E-05
Role of JAK family kinases in IL-6-type Cytokine Signaling	-	-	-	-	10	3.24E-05

5. Discussion

Variation in samples did not allow for conclusive statistical differences between silver-treated fish despite average tissue concentrations displaying noticeable trends. However, silver accumulation in silver-treated fish was statistically greater than controls. Despite the high variability in samples, the accumulation patterns still provide insight into accumulation differences between the Ag^+ ion and AgNPs. Accumulation was the greatest in the GI tract followed by liver, then gill and skin. While no silver accumulated in the brain above the limit of detection in our adult FHMs, previous studies have visually confirmed the presence of AgNPs in the brain of larval Japanese medaka (*Oryzias latipes*) after 21 d of exposure (Kwok et al. 2012). After brain, skin had the lowest tissue concentrations with no accumulation pattern that would indicate a unique target of AgNP exposure.

In liver, accumulation was similar between the high doses of all silver treatments despite greater total Ag water concentration, probably due to both brachial and intestinal uptake of Ag^+ ions. Similarly, in rainbow trout (*Oncorhynchus mykiss*) while initially the greatest accumulation was in GI tract and gill, by day eight the accumulation was redistributed to the liver (Galvez et al. 2002). The accumulation in liver at 96 hr could be attributed to the Ag^+ ions released from AgNPs revealing no unique particle effects in this organ.

The gill is the primary site of Ag^+ uptake and Na^+ channels are the major site of toxicity (Morgan et al. 1997). Because of the uptake of Ag^+ at the gill, it is a target of

accumulation and distribution to other organs. This study showed that despite a lower exposure concentration of total Ag, $AgNO_3$ had greater silver accumulation. AgNPs are probably cleared from the gill by sloughing off mucus (Chapter 3) before Ag^+ are released while dissolved $AgNO_3$ as Ag^+ is readily absorbed by the gill. Toxicity and accumulation in the gill is a result of the Ag^+ ion, therefore the gill is not a unique biological target of AgNP exposure.

In the GI tract, a pattern was present with AgNP-exposed fish having the highest silver accumulation. In addition, GI tract:gill accumulation was around 20-fold higher in fish exposed to AgNPs. The 20-fold higher accumulation in GI is consistent with the higher exposure concentration of AgNPs versus $AgNO_3$ exposed fish. Therefore, the GI tract accumulation was related to the total silver dose as opposed to the Ag^+ dose as was the case in the other tissues. Thus, the GI tract can be considered a unique biological target of AgNP exposure. In the previously mentioned larval Japanese medaka study, AgNPs were visualized in GI tract tissue but not in surrounding mesentery indicating that the GI tract was not a site of AgNP uptake (Kwok et al. 2012). Conversely, carp (*Cyprinus carpio*) accumulated silver in a similar pattern as the FHMs after exposure to AgNPs (Gaiser et al. 2012). AgNP uptake also occurs *in vitro* by human CACO-2 intestine cells revealing the GI tract as a site of AgNP uptake (Gaiser et al. 2012).

Microarray analysis revealed unique differential gene expression in each silver treatment in gill tissue. Accordingly, hierarchal clustering of gene expression clearly sorted each exposed fish into their appropriate exposure group. However, due to similarities in pathway analysis it would be hard to conclude that the variation was due

to unique particle effects because some variation could be explained by the differences in Ag^+ ion dose levels. Many of the altered genes and pathways, including the most enriched pathway in all treatments, cell cycle control of chromosomal replication, were related to cell cycle, apoptosis, and cell proliferation. When these pathways are considered with our previously performed FHM studies at the same time point and similar exposure concentrations, these altered pathways are consistent with the histopathological alterations in the gill including regressive alterations such as mucous cell degeneration and epithelial desquamation and progressive alterations including hypertrophy and new cell generation in hyperplasia (Chapter 2).

$AgNO_3$-exposed fish had downregulated prolactin (-3.2-fold). Prolactin is important in mucus production. For example, in discus fish (*Symphysodon aequifasciata*) upregulation of prolactin in parental fish increased mucus production needed to feed their fry (Khong et al. 2009). In goldfish (*Carassius auratus*) prolactin injection increased number of mucous cells (Ogawa 1970), and similarly bluestreak cleaner wrasse (*Labroides dimidiatus*) had an increased number of mucous cells after prolactin treatment (Lenke 1991). Prolactin down regulation by $AgNO_3$ could explain an increase in degenerated mucous cells in silver-exposed fish in our previous study (Chapter 2).

In comparison to other microarray studies in fish exposed to AgNPs, gills of zebrafish had distinct gene expression profiles between AgNPs and Ag^+ ions with genes related to cell proliferation and apoptosis probably due to gill remodeling similar to the FHM study presented here (Griffitt et al. 2009). FHM liver and brain microarrays also

indicated that pathways involved in cell cycle, apoptosis, and oxidative stress were enriched by $AgNO_3$ and PVP-AgNP exposure (Garcia-Reyero et al. 2014).

Most of the acute toxicity generated in FHMs after 96 hr exposure to AgNPs can be attributed to the Ag^+ ion at the gill. However, a unique biological target of AgNP toxicity was discovered in this study: the GI tract. AgNPs could increase toxicity at the GI tract by releasing Ag^+ ions over time or by direct particle interaction with the tissue. This could be even more significant with low-dose long-term exposure that is more likely from environmental exposure. Future work should compare DEGs in gill and GI tract of fish exposed to $AgNO_3$ and AgNPs to understand if unique particle effects occur.

6. Acknowledgements

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CHAPTER 6. THE INFLUENCE OF DISSOLVED ORGANIC CARBON ON ACUTE LETHALITY IN ZEBRAFISH (*DANIO RERIO*) EXPOSED TO SILVER NANOPARTICLES AND SILVER NITRATE

1. Synopsis

This chapter describes methods used to understand the relationship of acute lethality of citrate or polyvinylpyrrolidone (PVP) coated silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$) to varying concentrations of dissolved organic carbon (DOC). The results were obtained to provide a data set supporting the Environmental Modifying factors Tool in NanoExPERT (https://nanoexpert.usace.army.mil/), a suite of tools developed by The Nanomaterial Risk Assessment Focus Area at ERDC to aid in environmental risk assessment.

2. **Background**

Due to the known toxicity of silver to aquatic organisms (Wood et al. 1999), an understanding of the possible risk associated with the increased use and subsequent release of AgNPs in the aquatic ecosystem is needed. While an increasing number of studies are currently available on the toxicity of silver nanoparticles (Bondarenko et al. 2013), few have established toxicity patterns relative to environmentally relevant alterations in water chemistry parameters (e.g., Kennedy et al. 2012; Kennedy et al. 2014; Harmon et al. 2014; Gao et al. 2009)

While laboratory exposures that consider AgNP toxicity only in a single water type are crucial to understanding the mechanisms of toxicity, proper assessment of risk requires consideration of complex environmental matrices in which AgNPs could be present.

NanoExPERT is a suite of tools developed by The Nanomaterial Risk Assessment Focus Area at ERDC that aims to predict potential environmental risks associated with nanomaterial use (http://nanoexpert.usace.army.mil/). While applications of NanoExPERT extend past the Department of Defense and can aid the safe development of consumer products, an expansion of toxicity data is needed to more accurately estimate environmental risks. One such need is to address uncertainties of AgNP toxicity in varying water chemistry parameters such as dissolved organic carbon (DOC), ions, and pH to provide more accurate toxicity predictions in actual environmental matrices.

The known toxicity of the Ag^+ ion and its identification as the primary cause of acute toxicity of aquatic organisms (Kennedy et al. 2010) makes identification of ion release potential vital to understanding AgNP toxicity. Aggregation and dispersion can also be affected by water chemistry parameters resulting in changes in expected toxicity (Gao et al. 2009; McLaughlin and Bonzongo 2012). One such water chemistry parameter that can modify nanosilver toxicity is the presence of DOC. Increased DOC decreases toxicity of AgNPs by stabilizing the particles in zooplankton (*Ceriodaphnia dubia*) (Kennedy et al. 2012) and in bacteria (*Pseudomonas fluorescens*) by complexion of Ag^+ ions (Hogstrand and Wood 1998). When fathead minnows or juvenile rainbow trout were exposed to ionic silver in water with increased DOC levels, the 96-h LC_{50}

values significantly increased in both species (Bury et al. 1999). While these studies establish that DOC presence can mitigate silver toxicity, greater understanding is still needed when considering various fish species and AgNP particle sizes.

In order to accurately predict the toxicity of AgNPs, laboratory-based exposures must take into consideration actual environmental conditions in which silver nanoparticles can be found. During a data compilation conducted by the Nickel Producers Environmental Research Association, the Rhine River was found to have a DOC of 2.8 mg/L. Similarly, the United States Geological Survey determined the Snake River in Yellowstone National Park, Wyoming had a DOC of 0.9 to 4.5 mg/L while Panther Creek had DOC between 1.1 to 4.6 mg/L (Mebane 2008). Whereas, the Suwannee River was determined to have a DOC between 2.3 and 45.7 mg/L (Gao et al. 2009). Based on what is naturally occuring in the environment as reflected by these four bodies of water an environmentally relevant DOC range would be 0 – 40 mg/L.

In this study, we aimed to improve estimates of aquatic toxicity of nanoparticles to fish by establishing median lethal concentrations (LC_{50}) of citrate and PVP-AgNPs (nominally 20 nm) in increasing but environmentally relevant DOC concentrations of 1, 5, 10, 20, and 40 mg/L in a common freshwater model, larval zebrafish (*Danio rerio*). The results were then integrated into the Environmental Modifying Factors Tool in the NanoExPERT tool suite.

3. Materials and methods

3.1 Fish source.

Zebrafish, AB line wild-type, were purchased from the Zebrafish International Resource Center (ZIRC, Eugene, OR, USA). The fish were kept under the protocol approved by the University of Mississippi Institutional Animal Care and Use Committee. Keeping with the guidelines, the fish were raised in an Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL, USA) with pH 7.0–7.5 and salinity of 60 ppm in zebrafish water. Each habitat was kept between 25℃ and 28℃ with a 14:10 light-dark cycle. The zebrafish were fed live brine shrimp (*Artemia* sp.) and TetraMin® Tropical Flakes (TertraMin, Blacksburg, VA) twice daily. The sexually-mature breeders selected had no signs of disease or deformities. Eggs were collected using a receptacle at the bottom of the habitat that allowed the eggs to fall into the egg reservoir, while preventing the zebrafish from interfering with the fertilized eggs (Fang et al. 2013).

3.2 Moderately hard water

Moderately hard water (MHW) was made according to US Environmental Protection Agency (USEPA) guideline 821-R-02-013 by adding 1.20 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080 g KCl to 19 L of deionized water, and the solution was aerated overnight. $CaSO_4 \cdot 2\ H_2O$ (1.2 g) was added to a separate 1 L of deionized water, and the solution was mixed until the $CaSO_4$ was dissolved completely. Once dissolved, the $CaSO_4$ solution was added to the 19 L of MHW and stirred and aerated continuously until well mixed and equilibrated for 24 hrs before use.

3.3 Silver

$AgNO_3$ was obtained from a commercial source (Sigma Aldrich, St. Louis, MO) and diluted to a working stock in nanopure water with a nominal concentration of 10 µg/mL. PVP-AgNPs and citrate-AgNPs were obtained from Nano Composix (San Diego, CA, USA) at a concentration of 1 mg/mL and a nominal size of 20 nm. Concentrated stock suspensions were prepared by sonicating the sample mixture in a water bath for 5 minutes and inverting multiple times to mix. Concentrated stocks were diluted with nanopure water to obtain a working stock with a nominal concentration of 40 µg/mL. Particle sizing was verified by Field Flow Fractionation (PostNova F-1000 symmetrical flow Field Flow Fractionation, St. Lake City, UT). The concentrations of silver used during the DOC exposures were confirmed with ICP-MS, and the LC_{50} calculations were performed using measured silver concentrations as determined by ICP-MS.

3.4 Dissolved organic carbon

The dissolved organic carbon, Suwannee River extract, was purchased from the International Humic Substances Society (IHSS, Atlanta, GA). A mass of 40 mg of Suwannee River extract was added to one liter of MHW and filtered through a 40 micron filter to remove the undissolved portion. The remaining mixture was then diluted to nominal concentrations of 1, 5, 10, 20, and 40 mg/L. DOC concentrations were verified by the USDA National Sedimentation Laboratory (USDA-NSL, Oxford, MS).

3.5 Zebrafish LC_{50} exposures.

After 48 hours post fertilization, hatched larvae were verified to be alive by heartbeat and placed one larvae per well in a 96 well plate. The exposure was started

with addition of nominal 1, 10, 20, 40, 60, 80, 100, and 200 µg/L of $AgNO_3$ or 400, 800, 1200, 1600, and 2000 µg/L in AgNP exposures.

For each exposure, 20 larval zebrafish were exposed to each condition (i.e., 20 zebrafish were exposed to a DOC 40 mg/L at an $AgNO_3$ concentration of 100 µg/L). After dosing the zebrafish, the 96 well plates were wrapped in aluminum foil in order to protect AgNPs from increased aggregation due to UV light (Cheng et al. 2011). The foil-wrapped plates were then placed in an incubator at 26℃. At 96 hours post fertilization (a total of 48 hours of exposure), the plates were removed from the incubator, examined under an Olympus BX-40 microscope (Olympus Americas, Center Valley, PA) and the number of dead fish were determined by the absence of cardiac activity and recorded. The acquired data was then input into the EPA's LC_{50} calculation program (http://sdi.odu.edu/model/lc50.php).

4. Results

All aqueous silver concentrations were confirmed by ICP-MS analysis (Table 6.1). PVP-AgNP Ag concentrations agreed closely with the nominal concentrations while citrate-AgNPs concentrations were lower than the expected nominal concentration. Actual DOC concentrations closely agreed with the expected nominal DOC concentrations (Table 6.2). Particle hydrodynamic sizing were 28±4 nm and 26±3 nm for citrate-AgNPs and PVP-AgNPs, respectively. All three silver treatments were the most toxic at 0 mg DOC/L with LC_{50} values of 36.1, 448, and 826 µg/L for $AgNO_3$, citrate-AgNPs, and PVP-AgNPs, respectively (Figure 6.1). Toxicity decreased in all

treatments as the DOC concentration increased. The lowest toxicity was measured at the highest DOC tested (40 mg DOC/L) with LC_{50} values of 106, 880, and 1670 µg/L for $AgNO_3$, citrate-AgNPs, and PVP-AgNPs, respectively.

Table 6.1. Nominal versus measured silver concentrations in water for experiments with DOC.

Nominal (µg/L)	Measured (µg/L)	
	PVP-AgNPs	Citrate-AgNPs
400	465±4	346±11
800	781±41	600±20
1200	1124±58	871±55
1600	1526±25	1126±59
2000	1915±30	1386±72

Table 6.2. Nominal versus measured DOC concentrations.

Note: measured DOC concentrations are 40% of the nominal concentration of natural organic matter powder added.

Nominal DOC (mg/L)	Measured DOC (mg/L)
40	38.6±1.2
20	22±0.9
10	11±1.5
5	6.6±0.2
2.5	2.3±0.3
0	1.4±0.3

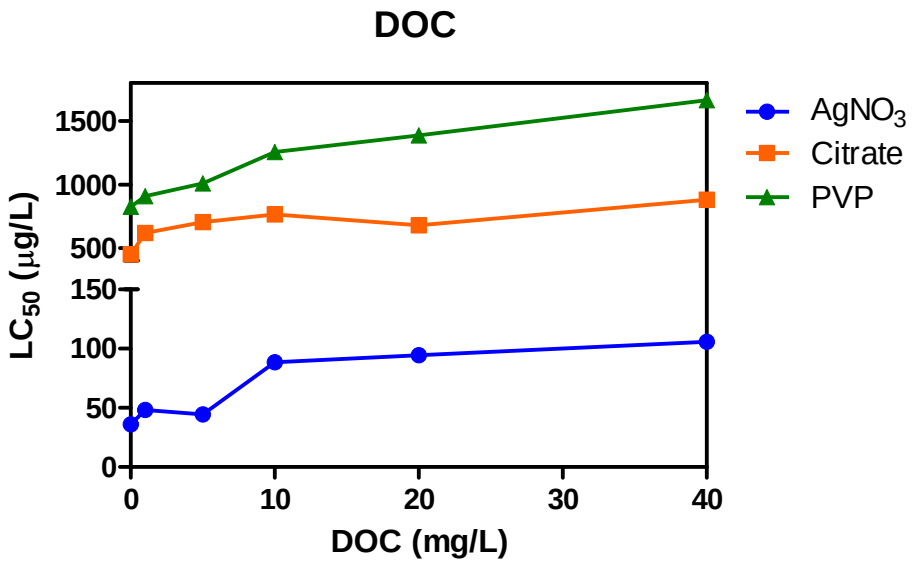

Figure 6.1. Lethal concentrations for 50% of organisms (LC_{50}s) after exposure to $AgNO_3$, citrate-AgNPs, or PVP-AgNPs in varying concentrations of DOC.

Silver toxicity is caused by the Ag^+ ion in aquatic organisms (Morgan et al. 1997). The toxicity of AgNPs is thought to be derived from the dissociation of free silver ions from particles (Newton et al. 2013; Kennedy et al. 2010). These free silver ions are produced as the equilibrium allows, which is determined in part by the water chemistry parameters of the aquatic environment. Therefore, there can be varying toxicities from AgNP particles of the same size and/or water concentration when water chemistry parameters are changed.

In embryo and larval fish, exposure to the Ag^+ ion and AgNPs has decreased swim bladder inflation, hatching and survival rates, and altered embryo activities (Powers et al. 2011; Powers et al. 2010). Developmental abnormalities in eye and central nervous system were observed after exposure to AgNPs in larval Japanese

medaka (*Oryzias latipes*) (Kashiwada et al. 2012) and zebrafish (Yeo and Kang 2008). Decreased hatching rate and survival were also common after AgNP exposure (Christen et al. 2013; Asharani et al. 2008; Powers et al. 2011)

In our study, as expected, the increased presence of DOC reduced the lethality of AgNPs and $AgNO_3$. The results were consistent with previous findings showing reduced toxicity in the presence of DOC in *Ceriodaphnia dubia* (Kennedy et al. 2012), *Pseudokirchneriella subcapitata* (McLaughlin and Bonzongo 2012), and *Daphnia magna* (Gao et al. 2012). While DOC coats AgNPs which increases stability due to steric hindrance (Kennedy et al. 2012; Fabrega et al. 2011), the relationship of toxicity reduction of AgNPs and DOC is unclear as DOC coating could prevent dissolution or reduce toxicity by complexation of released ions (Erickson et al. 1998).

4.1 Integration into NanoExPERT

Our data provides a relationship between AgNP toxicity and varying DOC concentration. While these data only provide a small picture of DOC and nanosilver interaction, utilization of these data combined with the work of others can lead to better risk prediction and understanding of toxicity. In order to make connections with other data and understand the broader picture, data must be combined in an easy to utilize central database. The NanoExpert tool suite (nanoexpert.usace.army.mil; Figure 6.2) was developed to support risk assessment of nanomaterials. Data resulting from these exposures contributed to the Environmental Modifying Factors Tool which helps explain the effects of changes in water chemistry parameters on toxicity in a range of organisms (Figure 6.3).

Further research is still needed to predict AgNP interactions with variations in other water chemistry parameters (e.g., varying pH and hardness) as actual aquatic ecosystems create a more complex scheme by which toxicity changes with multiple interactions occurring simultaneously.

Figure 6.2. NanoExPERT Tool Suite. Screen shot of NanoExPERT tool displaying the various tools available to aid in nanoparticle environmental risk assessment. Access: http:// nanoexpert.usace.army.mil.

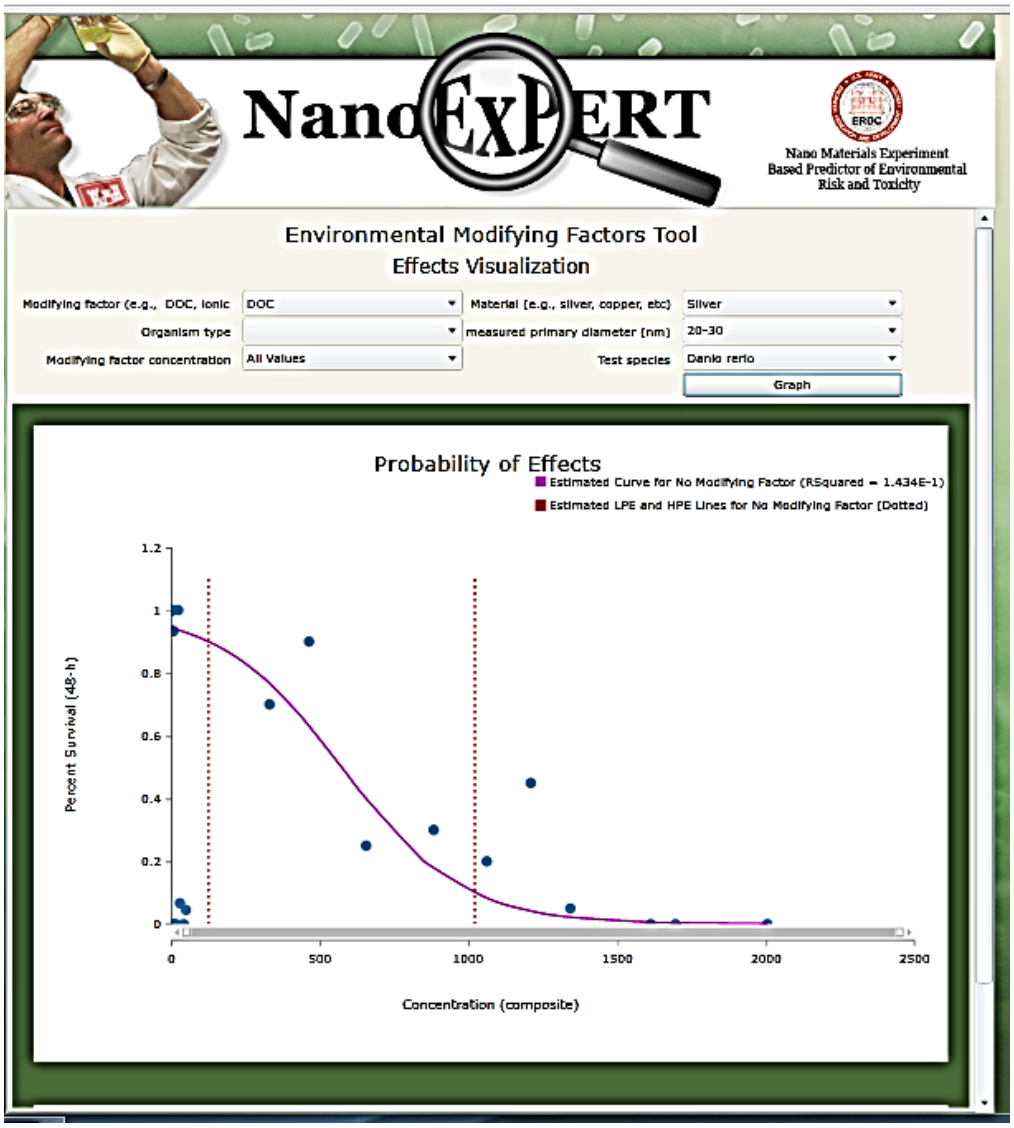

Figure 6.3. Environmental Modifying Factors Tool. Screen of DOC LC_{50}s in zebrafish from the modifying factors tool with the data from this study integrated.

5. Summary

The objective of this investigation was to determine mortality of larval zebrafish after exposure to $AgNO_3$, citrate-AgNPs, or PVP-AgNPs in varying concentrations of DOC. The results showed that increasing DOC decreased the toxicity of all silver formulations with LC_{50}s increasing from 36 to 106 µg/L in $AgNO_3$, 448 to 880 µg/L in

citrate-AgNPs, and 826 to 1670 µg/L in PVP-AgNPs when DOC was increased from 0 to 40 mg/L. The highest reduction in LC_{50}, up to 2.9-fold, was detected in the $AgNO_3$ treatments. These data were then integrated with other studies in the Environmental Modifying Factors Tool within the NanoExPERT tool suite to contribute to better environmental risk assessment of AgNPs in complex environmental matrices.

CHAPTER 7. CONCLUSION AND FUTURE DIRECTIONS

1. Conclusions

This research was performed to gain an understanding about the toxic effects of AgNP exposure as compared to the known toxic effects of the Ag^+ ion and establish unique toxicities and biological targets associated with AgNP exposure. Efforts were also taken to understand particle form upon uptake, patterns of AgNP accumulation and the changes in toxicity due to altered water chemistry parameters. This research can confirm the original hypothesis that the toxicity of AgNPs is mostly derived from the ionic portion, but that unique particle effects also exists and should be considered. The research aims of this project were also achieved with scientific advancements made including: the first identification of AgNPs in fish tissue after exposure using FFF-ICP-MS; the first utilization of a glucose surrogate method for mucus production in fish after exposure to a toxicant; and the first recognition of mucous goblet cells regression after silver exposure.

Aim 1. Identify changes in gill histopathology after exposure to silver nanoparticles (AgNPs) and silver nitrate ($AgNO_3$).

To understand changes gill histopathology FHMs were exposed to two concentrations of $AgNO_3$ (1.3 and 3.7 µg/L), citrate-AgNPs (15 and 39 µg/L), and PVP–

AgNPs (11 and 50 µg/L). Similar dissolved silver concentrations were achieved among the low doses of all treatments (0.2-0.3 µg/L) and high doses (0.8-1.1 µg/L) which allowed for the establishment of unique toxicities from AgNP exposure. When the sum of all histopathological abnormalities (weighted index) was calculated, all silver groups had a significantly higher index. While not significant, citrate-AgNPs had the highest index (10±0.32), and caused significantly increased specific alterations including hypertrophy, lamellar fusion, epithelial lifting, and desquamation. The percentage of regressed goblet cells was statistically increased in all silver treatments compared to control which suggested possible reduction in the ability of fish to produce mucus. Because the toxic mechanism of silver exposure is disruption of ionic regulation, the main ion transporter in gill, Na^+/K^+-ATPase, was assayed by visualizing immunoreactivity in tissue sections and a dose-dependent decrease in fluorescent signal was present in all silver treatments. These results indicated that toxicity was derived from the Ag^+ ion in both $AgNO_3$ and AgNPs treatments due to similar alterations of gill structure despite the higher total silver concentrations in the AgNP treatments.

Aim 2. Understand the effects of silver exposure on the fish mucus production.

Mucous cells were regressed after silver treatment in Aim 1, suggesting the limited ability to produce mucus. The mucus layer is present on all surfaces that interact with outside environment in fish and offers protection from xenobiotic exposure. While mucus hypersecretion is noted after initial exposure to xenobiotics, little is currently known about a fish's ability to continually produce protective mucus after subsequent exposure. In this work, mucus production ability was assessed for the first time after

silver exposure. FHMs were exposed to $AgNO_3$ (0.82 or 13.2 µg/L), PVP-AgNPs (11.1 or 208 µg/L) and citrate-AgNPs (10.1 or 175 µg/L) for 96 hr. Mucus concentrations based on glucose as a surrogate were determined at 0, 1, 2, 3, 4 and 24 hour after re-dosing each day. Our results confirmed the initial mucus hypersecretion after silver exposure on day 1. However, by day 4, mucus production was greatly reduced from the earlier hypersecretion on day 1 in silver-treated fish, while control fish produced consistent mucus concentrations throughout exposure. No unique particle effects were present in regards to mucus production. Reduction in protective mucus could lead to increased silver toxicity as well as increased sensitivity to other xenobiotics.

Aim 3. Identify unique biological targets of AgNP exposure.

Concerns about nanoparticles affecting different biological targets than their bulk counterparts arise because of their unique properties. To understand accumulation patterns of AgNP exposure compared to $AgNO_3$, FHMs were exposed to two concentrations of $AgNO_3$ (0.8 and 13 µg/L), citrate-AgNPs (10 and 175 µg/L), and PVP-AgNPs (11 and 208 µg/L) for 96 hrs. Skin, liver, GI tract, and brains were then acid digested and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for silver accumulation. AgNPs accumulated less than $AgNO_3$ in the gill, skin, and liver despite greater total silver water concentrations. Brain tissue had no silver accumulation above instrumentation detection limits in any silver formulation. AgNPs accumulated in the GI tract more than in the gill where ratios of concentrations (GI tract:gill) were 23:1, 17:1 and 0.44:1 for fish exposed to PVP-AgNPs, citrate-AgNP, and $AgNO_3$. This

differing pattern of accumulation indicates a possible unique biological target of AgNP exposure, the GI tract.

Aim 4. Characterize AgNPs in tissues of organism after uptake.

Understanding the toxicity of nanoparticles requires a greater understanding particle form as they interact with biological matrices. After establishment of a different potential biological target of AgNP, particle characterization in that tissue matrix offered a possible understanding of direct particle tissue interaction. While technically challenging, Field-Flow-Fractionation coupled to ICP-MS (FFF-ICP-MS) offered an opportunity to understand particle form in the tissue matrix. FFF-ICP-MS was utilized to characterize particles in GI tract and gill tissue after exposure to 171 or 212 µg/L of PVP-AgNP, and citrate-AgNP, respectively. PVP-AgNP exposed fish had particles agglomerated in the GI tract tissue with some particles found near the original hydrodynamic size of 26 nm but others at 40-70 nm, while gill tissue showed no particle agglomeration with a single fractogram peak sizing at 27 nm. In fish exposed to citrate-AgNPs, both GI tract and gill tissue displayed a single peak with AgNPs 40-55 nm range and 30 nm, respectively. The GI tract agglomeration pattern could be due to the relative stability of each AgNP particle coating in the water column prior to uptake and after ingestion. Sterically stabilized PVP-AgNPs are generally more stable in complex environmental media than the charge-stabilized citrate-AgNPs. These differences in stabilization could lead to homoaggregation of citrate-AgNPs and less interaction with biological substances or heteroaggregation in PVP-AgNPs and more interactions with biological substances forming a greater variety of sizes. The lack of AgNP aggregation

in the gill tissue was likely because particles present were from more recent exposure (e.g. within the last 24 hr of the 96 hr exposure) as compared to particles in the GI which may have accumulated throughout the exposure. The confirmed presence of AgNPs in GI tract is further evidence of a unique biological target of AgNP exposure.

Aim 5. Identify differential gene expression after exposure to AgNPs or $AgNO_3$.

Gill microarrays were performed after fish were exposed to $AgNO_3$ (5.7 µg/L), PVP-AgNPs (75 µg/L), or citrate-AgNPs (84 µg/L). There were 110 commonly differentially expressed genes (DEGs) between all silver treatments relative to control, and 98 common genes of AgNP exposure. However, $AgNO_3$ had 185 unique differentially expressed genes, PVP-AgNPs had 423 unique DEGs, and citrate-AgNPs had 615 unique DEGs. From the DEGs produced from silver exposures it was predicted that there were 26 common pathways differentially impacted by silver exposure regardless of formulation mostly to relate to apoptosis and cell cycle control. Citrate-AgNPs caused the highest number of pathway alterations (62), followed by $AgNO_3$ (31) and PVP-AgNPs (20). Gene expression and predicted pathways alterations were different between AgNPs and $AgNO_3$ exposed fish gills. However due the similarity in function of genes and pathways altered it is arduous to attribute differences in expression to unique particle effects because the changes could be due to different Ag^+ ion doses. Further analysis of genetic alterations in additional tissues, such as GI tract, could divulge unique particle effects and a better understanding of AgNP toxicity.

Aim 6. Understand the influence of DOC on AgNP and silver nitrate toxicity.

Changes in water chemistry can alter the toxicity of AgNPs. Laboratory exposures are often not representative of actual aquatic ecosystems due to the difficulty in recreating the complex natural environment. To properly predict risk of AgNP exposure, an understanding of changes in toxicity as influenced by different water chemistry parameters is needed. To partially address this challenge, zebrafish (ZF) larvae were exposed to concentrations of 1, 10, 20, 40, 60, 80, 100, and 200 µg/L of $AgNO_3$ or 200, 400, 800, 1000, 1200, 1400, and 1600 µg/L of PVP-AgNPs and citrate-AgNPs from 48 to 96 hours post fertilization in increasing environmentally relevant concentrations of DOC (0-40 mg/L). Deaths in each treatment were determined by lack of cardiac activity and the median lethal concentration (LC_{50}) was determined for each experimental condition using the EPA's LC_{50} calculation program. As expected, ionic silver as $AgNO_3$ was more toxic than either of the AgNPs tested. Increasing DOC decreased silver lethality in all treatments. The results from these exposures were then integrated in the NanoExPERT suite of tools. This data was added to the modifying factors tool which will help address uncertainties of AgNP toxicity in varying water chemistry parameters and provide more accurate risk assessment.

2. Future directions

2.1 Further understanding of the GI tract as a biological target.

Data from this study suggests that the GI tract of FHMs is a unique biological target of AgNP exposure. While AgNPs accumulate in greater concentrations than ionic silver and particles were confirmed to be present in the GI tract, further information is

needed to confirm unique toxicities from AgNPs. One suggested approach could be to understand alterations in gene expression by RNA microarray analysis and then assess whether unique molecular pathways are affected in the AgNP verses the $AgNO_3$ treated fish tissues.

2.2 Better characterization of AgNP chronic toxicity.

AgNPs have the ability to release silver ions into the environment or tissue at a slower rate. While the slow release of ions may reduce acute toxicity when compared to similar concentrations of ionic silver, an increase in sublethal toxicities could occur. Much of the regulation and risk assessment is based on the acute toxicity of Ag^+ ions, so concentrations of AgNPs that are considered harmless (e.g. below regulatory values) could actually produce toxicities over time. Longer term toxicity testing of AgNPs need to be performed to understand if AgNP regulations are needed and/or the total silver regulations need to be adjusted based on increased AgNP chronic toxicity. The knowledge gap in chronic toxicity data, could lead to an underestimation of risk and allowance of toxic concentrations of AgNPs to enter the aquatic ecosystem.

2.3 Further understanding of AgNP dissolution kinetics.

In order to further understand how alterations in toxicity are caused by variation in water chemistry parameters, experiments need to be performed to understand dissolution of AgNPs in the presence of different concentrations of DOC. Dialysis

membranes, which allow free Ag+ ions to evacuate but retain AgNPs, can be used to quantitate dissolution in various water chemistries. One product utilizing a dialysis membrane is the Float-A-Lyzer® G2 device which features an ultra-pure biotech cellulose ester membrane with a greater than 95% sample recovery (Spectrum 2013).

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VITA

Name Adam D. Hawkins

Address Environmental Toxicology Research Program

310 Faser Hall, University, MS, 38677

Phone 662-915-7612 (work); 706-295-5003 (cell phone)

E-mail adhawkin@go.olemiss.edu or adamhawkins01@gmail.com

Education

2008 – 2010 Reinhardt College, GA, USA

B.S. in Biology with honors, GPA 3.55

Thesis: Pan1 as a PAK Protein.

2005 – 2008 Georgia Highlands College, GA, USA

A.S. in Biology.

Professional Memberships

2010- Member, Society of Environmental Toxicology and Chemistry (SETAC)

2011- Member, Society of Toxicology (SOT)

Publications related to dissertation research

1. **Hawkins A.D.,** Thornton C., Kennedy A.J., Bu K., Cizdziel J., Jones B.W., Steevens J.A., and Willett K.L. Gill histopathologies following exposure to nanosilver or silver nitrate. In Preperation.

2. **Hawkins A.D.,** Thornton C., Steevens J.A., and Willett K.L. Alteration in *Pimephales promelas* mucus production after exposure to nanosilver or silver nitrate. Submitted.

3. **Hawkins A.D.,** Bednar A.J., Bu K., Cizdziel J., Steevens J.A., and Willett K.L. Identification of silver nanoparticles using flow field flow fractionation in *Pimephales promelas* gastrointestinal tract and gill tissue. Submitted

4. **Hawkins A.D.,** Guyton W., Stanley, J.K, Kennedy A.J., Steevens J.A., and Willett K.L. The influence of dissolved organic carbon on acute lethality in zebrafish (*Danio rerio*) exposed to silver nanoparticles and silver nitrate. Submitted.